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The Impact of Temperature on the Early Stages of the Development of the Body Axis in the Model Species Astyanax mexicanus (Teleostei: Characidae)

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The Impact of Temperature on the Early Stages of the Development of the Body Axis in the Model Species *Astyanax mexicanus* (Teleostei: Characidae)

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Abstract

Environmental fluctuations can change the phenotypic traits of ectotherms. Ectotherms such as fishes are very susceptible to changes in temperature. Recent studies on adults of the Mexican tetra, *Astyanax mexicanus*, indicate that variation in temperature during development significantly impacts vertebral number and body shape. Since vertebral number and somite number are related, I examined whether temperature significantly impacts somitogenesis and the early development of the body axis in this species. Fertilized eggs of the surface form of lab-reared *Astyanax mexicanus* were subjected to temperature treatments of 20°C, 24°C, and 28°C, and fixed hourly as they developed until they hatched. Embryos were stained with DAPI and imaged using a confocal microsope. Body length, somite number, unsegmented tail length, and somite length were measured.

Temperature significantly influenced the general rate of somitogenesis and all phenotypic traits measured. Surprisingly, temperature significantly influenced the unsegmented tail length even when variation in general developmental rate was accounted for, such that embryos reared at 24°C had a significantly longer unsegmented tail length than embryos reared at 20°C and 28°C. Although somite length was variable between developmental stages and along the body axis, temperature also seemed to influence somite length. For example, embryos reared at 28°C had consistently shorter posterior somites at the 40 somite stage of development than embryos reared at 20°C or 24°C. Understanding whether these temperature effects on somitogenesis also influence the phenotypic properties of adults is a major avenue of future research.

This study provides a better understanding of how temperature influences the early stages of the development of the body axis in this emerging model species. It will also provide a baseline for future studies examining the influence of phenotypic plasticity on body form variation in characids colonizing new habitats.

CHAPTER I

Literature Review

In this chapter, I provide a review of the literature on background topics related to my research including phenotypic plasticity and its potential role in adaptive evolution, the establishment of the body axis in vertebrates, the factors influencing vertebral number and body form variation in fishes, and the emergence of *Astyanax mexicanus* as a model system in evolutionary developmental biology,

Phenotypic Plasticity and Evolution

Understanding how genotypes respond to changes in the environment is one of the main goals of biology. Different factors can impact the morphological traits of animals in their habitats including genetic variation and environmental factors like temperature fluctuation, nutrition, abiotic and biotic stressors, etc. (Beacham & Murray, 1986; Carroll, 1995; Hoffmann & Hercus, 2000; Bosch et al., 2014; Schulte, 2014; Kulkarni & Laender, 2017). Also, changes in the environment can have a direct influence on organisms that triggers changes in their behavior, physiology, and morphology. These changes are known as phenotypic plasticity (Price et al., 2003). Phenotypic plasticity is the ability of a genotype to produce different phenotypes when exposed to changes in environmental conditions (West-Eberhard, 1989). Phenotypic plasticity is common in animals and plants and is crucially important for the survival of many lineages in nature given the unpredictable fluctuations in environmental conditions that they face (Agrawal, 2001; Atkin et al., 2006; Fusco & Minelli, 2010; Gotthard & Nylin, 2016; Stevens, 2016;

Scheepens et al., 2018). Phenotypic variation caused by environmental factors can lead to phenotypic accommodation, which is the ability of an organism to make an adaptive adjustment in their phenotype without changes to their genome (West-Eberhard, 2003).

Phenotypic plasticity has been studied in different organisms to understand the mechanisms at play. For example, predators such as piscivorous fishes have an impact on young fishes. Young perch and roach show morphological changes in the presence of Pike, with perch increasing their body depth and roach showing displacement of dorsal and pelvic fins and changes in the width of the anal fin (Eklöv & Jonsson, 2007). Similarly, snails from different sites increased shell thickness when exposed to predatory crabs (Trussell, 1996). Diet, development, age, and family all influenced changes in the shape of the head, body, tail, and fins in two species of the cichlid *Geophugus* (Wimberger, 1992). Daphnia produced neck teeth in the presence of the predatory glassworm *Chaoborus* (Lüning, 1992) while they did not grow a neck spine when reared in the presence of a predatory backswimmer, but they were smaller than the control treatment (without predator) because the latter predator favored large prey (Dodson & Havel, 1988). Given its importance in nature, there is currently much interest in understanding the extent to which patterns of phenotypic plasticity influence the evolutionary potential and trajectories of lineages (Agrawal, 2001; Arthur, 2002; West-Eberhard, 2003; Crispo, 2007; Wund et al., 2008; Pfennig et al., 2010).

Phenotypic plasticity can be an adaptation to environmental changes such as the presence or absence of predators, seasonal fluctuations, and population density (Gibert, 2017). Phenotypic plasticity can also eventually lead to genetic assimilation. Conrad Waddington defined genetic assimilation as a phenotypic change that starts off being the

product of an environmental influence and eventually becomes a genetically fixed trait. Thus, the mutant phenotype that is expressed in response to some changes in the environment, can be selected for and passed down to future generations, eventually being expressed whether the environment stimuli is present or not (Waddington, 1953; West-Eberhard, 2003). For example, Waddington studied the effect of the environmental changes on *Drosophila* by inducing heat shock to the larvae in one group and non-heat shock to the other group to examine if the heat shock will produce different phenotypes. Some individuals in the heat shock treatment produced cross-veinless wings, but the other treatment did not produce the mutant phenotype. He also created two lines of the normal wings phenotype and cross-veinless wings phenotype to examine the effect of the heat shock. He kept heat-shocking larvae and kept selecting for 23 generations. After several generations, he noticed a few flies developed the cross-veinless wing phenotype in the absence of the heat shock. He also noted that the frequency of the cross-veinless phenotype increased over generations (Waddington, 1953; Crispo, 2007).

Phenotypic plasticity can play an important role in the adaptive process and promote individual survival during periods of rapid environmental change in the wild. Genetic variation and different environments can produce a range of variable phenotypes in populations with the beneficial plastic phenotypes forming the basis for long-term survival in the new environment.

Temperature fluctuations of water are one of the major environmental factors affecting aquatic ectotherms like fishes, which cannot sustain a steady body temperature through homeostatic mechanisms (Johnston, 2006; McDowall, 2008; Tsoukali et al., 2016; Boltaña et al., 2017; Kuczynski et al., 2017). A study conducted by Boltaña et al.

(2017) provided evidence that fish are impacted by temperature fluctuations during their development, which modifies their metabolic rate, growth, muscle defects, and physiology. In addition, other factors associated with temperature variation such as time of migration and reproduction, fertility, survival, and maturation can be affected (Crozier & Hutchings, 2014). Previous research on the impact of temperature variation on body form and the vertebral column in fishes found that vertebral number, the ratio of abdominal to caudal vertebrae and body shape are all significantly influenced by temperature (Reyes Corral & Aguirre, 2019). Thus, temperature must also influence the expression of the genes involved in the development of these traits. Understanding how temperature affects the development of the body axis and associated genes can provide insight into evolutionary history and potential of lineages.

Establishment of the Vertebrate Body Axis

The formation of the vertebrate body axis is an important process during embryogenesis. Embryogenesis is the process of cell proliferation, division, and differentiation that occurs during the early development of embryos. During gastrulation three different germ layers are formed: the ectoderm, mesoderm, and endoderm. Mesoderm, which is the middle layer, is an important layer in the formation of the vertebrate body axis. The pre-somatic mesoderm (PSM) is a precursor of the paraxial mesoderm that proliferates towards the posterior axis. The PSM is undifferentiated tissue that starts to differentiate during the development and the formation of embryos and gives rise to somites in a process known as somitogenesis. The formation of somites starts early in embryogenesis by increasing the size of the two parallel tissues of the PSM and

differentiating the embryonic PSM into repeated pattern of segments along the body axis in an anterior to posterior direction (Fig. 1) (Schröter et al., 2008; Maroto et al., 2012; Hubaud & Pourquié, 2014a). Somites are formed in a highly controlled manner and arranged in pairs along the body axis. Somites give rise to vertebrae, ribs, skeletal muscles, and the dermis of the back (Bénazéraf & Pourquié, 2013). While the PSM tissues continues to differentiate into new somites anteriorly, cell proliferation continues posteriorly, allowing the elongation of the body axis (Bénazéraf & Pourquié, 2013). The regulation of somitogenesis and axis elongation determines the number of segments and the length of the body in embryos.

The segmentation process is a fundamental process that occurs in all vertebrates. The axial skeleton is similar in its basic structure in all vertebrates and plays an essential role in supporting the body, while also allowing flexibility of movement (Maroto et al., 2012). The number of the body segments and the size of the PSM is controlled by the relationship between somitogenesis and the speed of axis elongation (Bénazéraf & Pourquié, 2013). Therefore, the final segment number in each embryo depends on two different factors (Gomez and Pourquié, 2009). First, the regulation of the posterior proliferation of cells during the process of somite formation (a long body has more somites). Second, segment-size plays a role in making an organism have more or less somites (small segments lead to more somites and large segments lead to less somites).

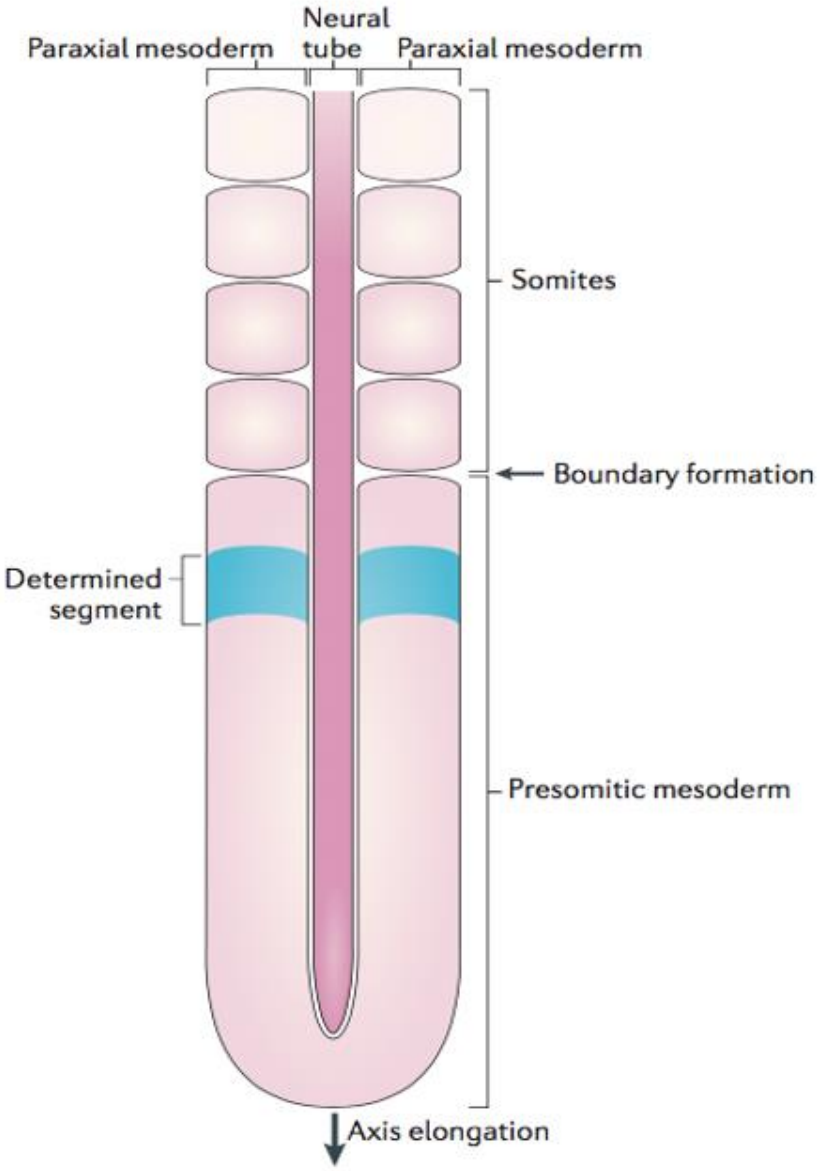

Figure 1: The neural tube in the center of the pre-somatic mesoderm (PSM) divides the tissue into two bilateral tissues called paraxial mesoderm. The undifferentiated PSM tissue starts to differentiate into two pairs of somite along the axis of the embryos. It segments the body from the anterior towards the posterior regions. The blue square indicates the determination front where the future somite will form (Hubaud & Pourquié, 2014a).

The formation of segments is thought to be controlled by a mechanism called the clock and wave-front model (Dubrulle & Pourquié, 2004). The segmentation clock is driven by a molecular oscillator that controls the repeated periodic formation of segments. Thus, the molecular oscillator operates the periodic activation of genes like

Notch, *Wnt*, and *FGF* in the PSM that result in cyclic waves of gene expression (Hubaud & Pourquié, 2014b). Cyclic genes are first expressed in the posterior or caudal part of the PSM, then move into the intermediate part, and finally into the anterior part of the PSM. The cyclic waves of gene expression will be repeated several times until the end of the formation of somites (Bénazéraf & Pourquié, 2013). The waves of gene expression induced by the clock genes result in the formation of repeating somites (Bénazéraf & Pourquié, 2013). Somite formation is specifically controlled by *Wnt* and *FGF* signaling pathways that move in a posterior to anterior direction, interacting with *RA*, which comes from the previously formed somites in an anterior to posterior direction. The interaction between these genes result in a determination front, which determines where the somites will form. Therefore, the segmentation clock induces a signaling rhythm along the body axis of the embryo resulting in the patterning of the PSM tissue into segments. Each time a new somite is formed, the signaling rhythms carry waves of gene expression that sweep across the body axis. Once the determination segment is detected, it will start forming the somite and continue to form new somites along the body axis (Fig. 2) (Hubaud & Pourquié, 2014a). Another gene that regulates the size and shape of somite is *Mesp2* which stands for mesoderm posterior protein 2. The expression of *Mesp2* is a major element in merging the cyclic and the determination front signals, which stimulates signal cascades that determine the segmentation boundaries. Thus, when cells pass the wave genes and the determination front, the expression of *Mesp2* will be activated and used as a template for the next somite (Fig. 3) (Gomez and Pourquié, 2009). The formation of somites will continue to segment the PSM along the axis until the PSM is exhausted, which leads to the termination of somitogenesis.

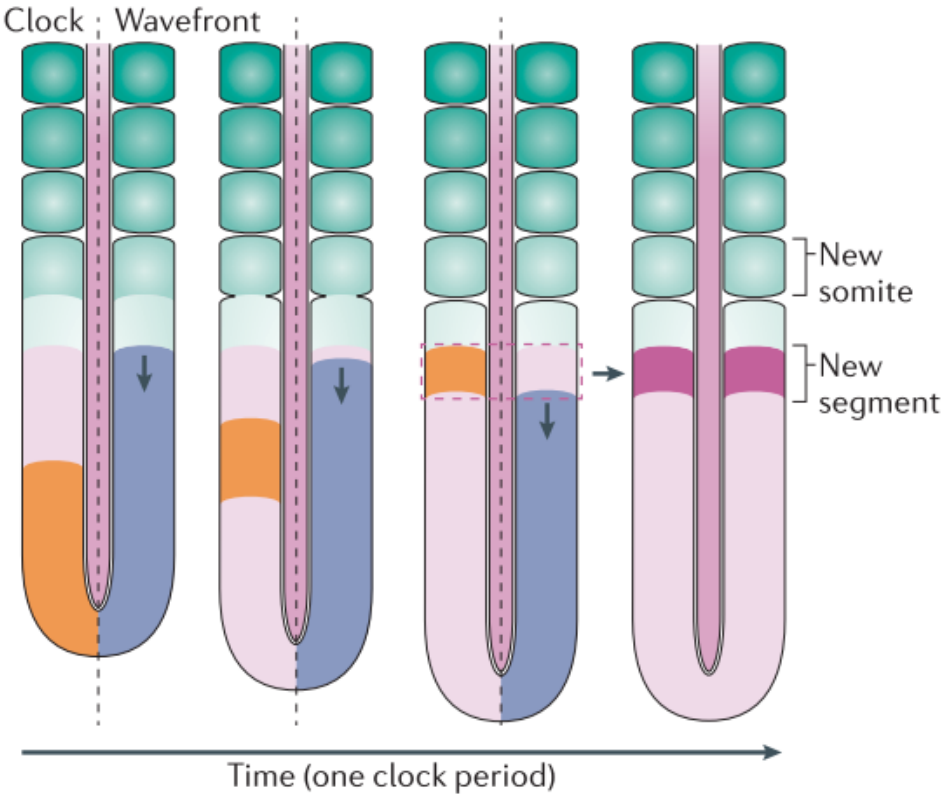

Figure 2: The clock and wave-front mechanism and the formation of the somites. Somite number increases over time (Hubaud & Pourquié, 2014a).

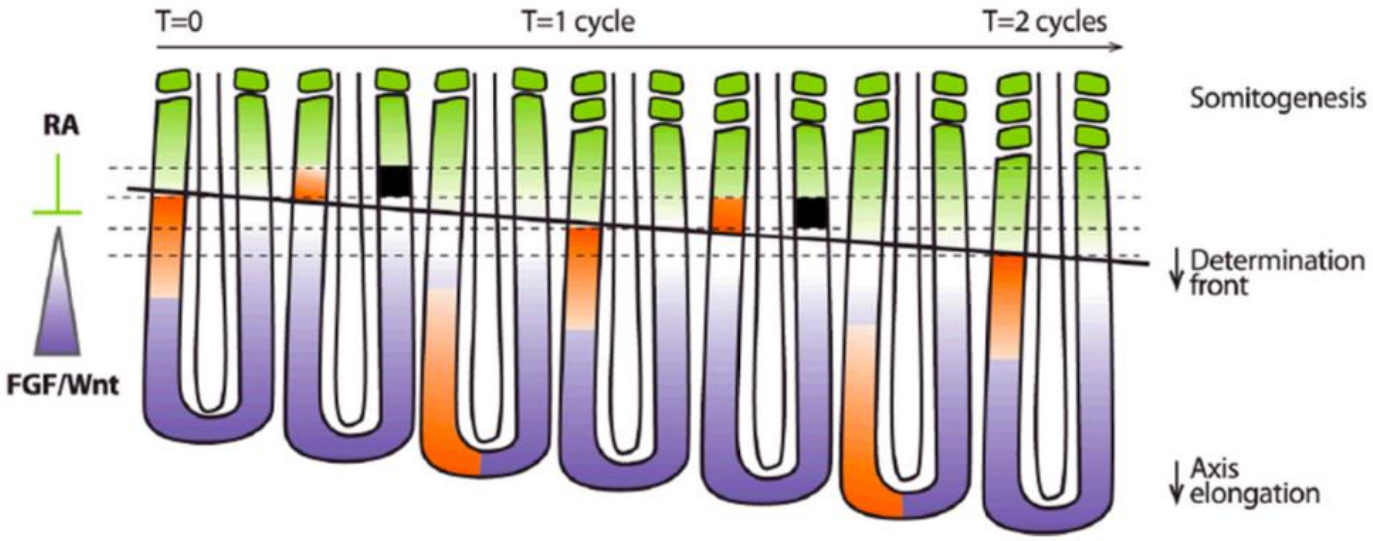

Figure 3: The cyclic genes *Wnt/FGF* (purple) express along the body axis from the posterior to the anterior direction. The wave front (orange) is expressed after the expression of *Wnt/FGF*. Thus, the somite will form after the expression of both the cyclic genes and wave front. The expression of *RA* down regulates the expression of *Wnt/FGF, which* leads to the termination of somitogenesis. In addition, the expression of *Mesgp2* (black square) will be activated after the cyclic genes and the determination front have passed across the PSM (Gomez and Pourquié, 2009).

Somitogenesis terminates with a continuous shrinking of the PSM in the tail bud. The *Wnt/FGF* signaling pathway is down-regulated in the tail bud region compared to the earlier stages of this process. Shrinking the PSM brings the *RA* signaling pathway near the tail bud region, which down regulates the *Wnt/FGF* pathway that stimulates body axis growth. This mechanism may also stimulate cell death (Fig. 4) (Bénazéraf & Pourquié, 2013). The number of somites that embryos achieve relies on the length or lifetime of PSM during the development of embryos.

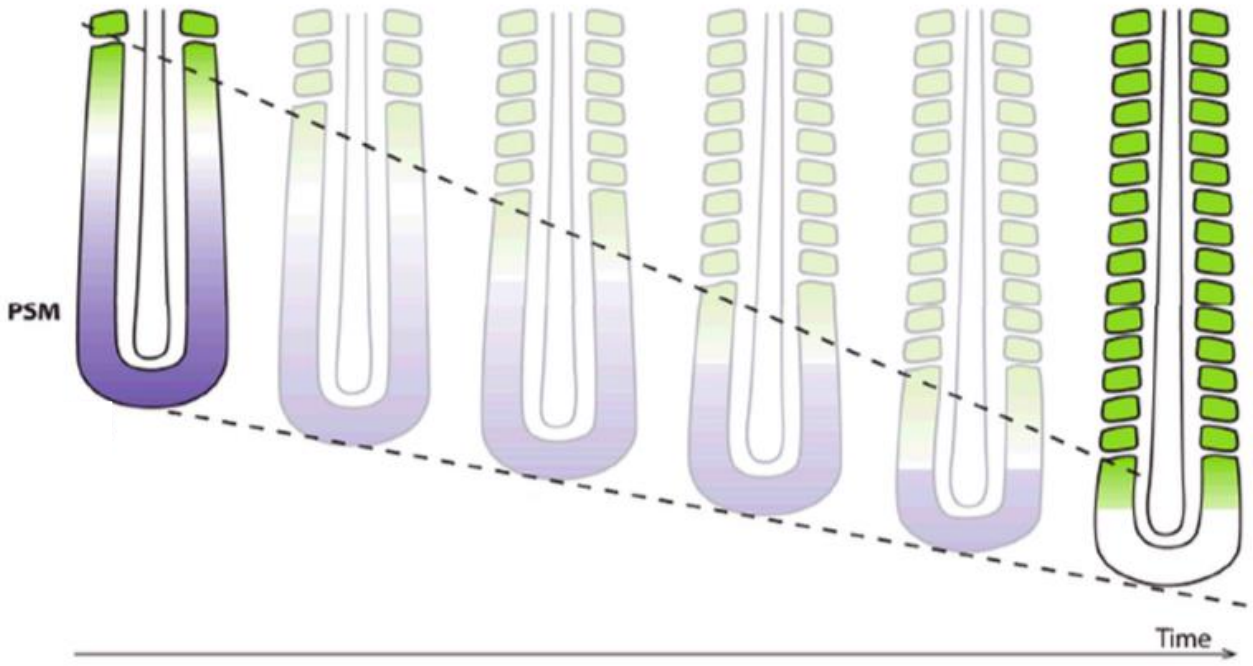

Figure 4: The ratio between the somitogenesis speed and the axis growth. When somitogenesis period or speed increases, the PSM tissue decreases. The shrinking of the PSM brings *RA* (Green) closer to the tail bud region, which down regulates the *Wnt/FGF* signaling pathways and leads to the termination of somitogenesis (Gomez and Pourquié, 2009).

The number of somites varies among species (Gomez et al., 2008). Gomez et al., (2008) examined somitogenesis in zebrafish, chicken, mouse, and corn snake embryos to understand how somite number is established in morphologically distinctive species. The corn snake was included because it forms many more somites than the other three species. Genes that regulate the formation of somites were stained with probes across different species to examine their expression via *in situ* hybridization. The results showed that in all four species there is a similar clock and wave front mechanism that regulates somitogenesis. Gomez et al., (2008) also investigated the differences in these periods with the general development rate for each species. They compared the time that the corn snake and chicken would take to develop the head. The morphology of the head in snake

embryos at the 230 somite stage looks similar to the head of the chicken embryo at the 40 somite stage (3 days), which indicates the slowing of the general development rate of the snake embryos compared to the other species. This indicated that somite formation is greatly accelerated in snakes relative to the overall development of the body, resulting in a large number of somites and vertebrae in snakes.

More detailed work on the effect of temperature on somitogenesis in fishes has been conducted in zebrafish, the major model fish system in biology (Schröter et al., 2008). Schröter et al., (2008) examined somitogenesis period (time for somite formation), somite length, and the wavefront speed at 20°C, 24°C, and 30°C. They found that somitogenesis period relies strongly on temperature treatments and that somite number maintains a linear relationship with time at different temeprature treatments. Fish reared at high temperatures take less time to form somites compared to fish reared at low temperatures. However, the somitogensis period varied along the body axis, being constant in the trunk and increasing in the tail bud region. They also found that somite length did not differ between temperature treatments. Somite length is greatest in the middle of the trunk and somites are shorter in the anterior and tail bud regions across all temperature treatments.

Factors Influencing Vertebral Number and Body Form Variation in Fishes

Fishes are the most diverse group of vertebrates with more than 500 families and approximately 30,000 species (McDowall, 2008). With this taxonomic diversity, comes great morphological variation. Vertebral number is among the most important and variable morphological traits in fishes. Vertebral number is functionally important,

influencing how fish swim, and is also used in fish taxonomy (Ward & Mehta, 2010; Ackerly & Ward, 2016). The number of vertebrae in fishes is highly variable among species and within the same species. It varies from 16 vertebrae in sunfish to 750 vertebrae in some eels (McDowall, 2008). Different factors such as body size, body shape, latitude, and temperature are associated with variation in the number of vertebrae in fishes (McDowall, 2008). For example, fish with elongated bodies are known to have high vertebral numbers (Ward & Brainerd, 2007; Ward & Mehta, 2010).

Temperature is one of the fundamental factors influencing vertebral number in fishes (McDowall, 2008; Reimchen & Cox, 2015; Ackerly & Ward, 2016). Most aquatic vertebrates are ectotherms and are thus vulnerable to the effects of fluctuating temperatures during early embryogenesis (Johnston, 2006; Ayala et al., 2015; Reyes Corral & Aguirre, 2019). The relationship between temperature variation and vertebral number was first reported by Jordan (1892) and is known as Jordan's rule or the law of vertebrae. According to Jordan's rule, the number of vertebrae in fishes increases with high latitude (low temperature) and decreases at low latitudes (high temperature). Although the details of the relationship vary among species, a general relationship between temperature and vertebral number has been reported repeatedly in fishes (reviewed in McDowall, 2008), including recently in *A. mexicanus* (Reyes Corral & Aguirre, 2019)

Astyanax mexicanus as an Emerging Model System in Evolutionary Developmental Biology

Astyanax mexicanus, the Mexican tetra, has become one of the major model systems in developmental biology, especially in evolutionary development (evo-devo), because of the extraordinary levels of phenotypic divergence that it exhibits. *Astyanax mexicanus* is in the order Characiformes, a hyper-diverse group of freshwater fishes with approximately 1,800 species, and in the family Characidae, which is the most diverse family of freshwater fishes in South and Central America with approximately 1,200 species (Mirande, 2010). Characids differ greatly in their ecology, size, body shape, and in many features of their internal skeleton (Mirande, 2010; Pereira et al., 2011; Escobar-Camacho et al., 2015)

Astyanax mexicanus is a particularly interesting species that includes two highly divergent ecomorphs: the eyed and pigmented surface population and the blind and unpigmented cave population, which evolved from the surface form (Hinaux et al., 2011). The cave populations diverged over a million years ago from surface ancestors and evolved specific changes in their morphology, physiology, and behavioral traits in response to the selective pressures in the cave environments. Due to the severe environmental conditions such as permanent darkness and food deficiency in their habitat, cavefish have lost their eyes and body pigments (Hinaux et al., 2011). The extreme morphological difference between the surface and cave populations is mostly genetic and likely the result of adaptive evolution. There are at least 29 different cave populations and these have evolved independently at least four times from the surface

form (Coghill et al., 2014). Despite the morphological differences between morphs, they are readily crossable in the lab, which facilitates inference of the genes and developmental mechanisms responsible for their divergence (Jeffery, 2001; Borowsky, 2008; Hinaux et al., 2011; Gross et al., 2015). As a result, *A. mexicanus* is a good system to study the developmental basis of adaptive evolution (Jeffery, 2001; Borowsky, 2008). Knowledge gained from it may provide insight into the genetic and developmental basis of morphological variation seen in other genera in the diverse family Characidae and possibly other organisms as well.

Previous research on lab-reared fish of the surface morph of *A. mexicanus* indicates that it exhibits significant phenotypic plasticity in body form and its axial skeleton in response to temperature changes during development. Reyes Corral and Aguirre (2019) found that fish reared at lower (20°C) and higher (28°C) temperatures had more vertebrae than fish reared at intermediate temperatures (23 and 25°C), and the ratio of precaudal to caudal vertebrae declined with temperature. Body shape was also impacted by temperature variation and covaried with vertebral number, with fish having more vertebrae generally being more streamlined than those with less vertebrae. When these differences develop is not clear since the fish in the study were several months old and already exhibited differences by the time data collection began. Thus, *A. mexicanus* may also be a promising system in which to study the effects of temperature changes on body form and the axial skeleton.

Finally, there are also practical reasons that make *A. mexicanus* a good model species for laboratory research (Jeffery, 2001). Jeffery explained the features that made *A. mexicanus* a good model system in evolutionary development. Many are similar to the

features that make zebrafish a good model system in biology. These include that they are easy to keep and cross in the lab, females produce large numbers of eggs that are fertilized externally by the male, the eggs and embryos are transparent, which facilitates studying their development, embryonic development is extremely fast, faster even than zebrafish, with eggs hatching within 24 hours at standard rearing temperatures, etc. A developmental staging table for *A. mexicanus* reared at standard temperatures and the complete genome are also available (Jeffery, 2001; Borowsky, 2008; Hinaux et al., 2011; Gross et al., 2015).

CHAPTER II

Introduction

Temperature is one of the most important factors influencing the development of ectothermic organisms (McDowall, 2008; Reimchen & Cox, 2015; Ackerly & Ward, 2016). Most aquatic vertebrates are ectotherms and are thus vulnerable to the effects of fluctuating temperatures during early embryogenesis (Johnston, 2006; Ayala et al., 2015; Reyes Corral & Aguirre, 2019). Changes in temperature during development are known to affect key properties of the bodies of fishes including serially repeating elements like vertebrae. For example, the relationship between temperature variation and vertebral number was reported by David Star Jordan as early as 1892, and is known as Jordan's rule or the law of vertebrae. According to Jordan's rule, the number of vertebrae in fishes increases with high latitude (low temperature) and decreases at low latitudes (high temperature). Although the details of the relationship vary among species, a general relationship between temperature and vertebral number has been reported repeatedly in fishes both in studies examining patterns of natural variation and experimental lab studies (McDowall, 2008). However, the developmental mechanisms through which changes in temperature affect vertebral number are not known. Vertebral number is directly related to the number of somites that form during embryogenesis (Fleming et al., 2015), so it is possible that temperature is having an effect very early in development when the body axis is forming and segmenting, well before the vertebrae actually form.

The formation of the vertebrate body axis and the segmentation of the body are important processes that occur during embryogenesis. Embryogenesis is the process of cell proliferation, division, and differentiation, which occurs during the early

development of embryos (Richardson et al., 1998; Stickney et al., 2000; Morin-Kensicki et al., 2002; Gomez and Pourquié, 2009). During gastrulation three different germ layers are formed: the ectoderm, mesoderm, and endoderm. The mesoderm, which is the middle layer, plays an important role in the formation of the vertebrate body axis. The pre-somatic mesoderm (PSM) is a precursor of the paraxial mesoderm that proliferates towards the posterior axis. The PSM is undifferentiated tissue that starts to differentiate during the formation of the embryo's body and gives rise to somites in a process known as somitogenesis. Somites are blocks of tissue located on both sides of the vertebrate neural tube during embryogenesis that give rise to vertebrae, ribs, skeletal muscles, and the dermis of the back (Bénazéraf & Pourquié, 2013).

The formation of somites is tightly controlled and starts early in embryogenesis by increasing the size of the two parallel tissues of the PSM and differentiating the embryonic PSM into repeating segments along the body axis in an anterior to posterior direction (Schröter et al., 2008; Maroto et al., 2012). While the PSM continues to differentiate into new somites anteriorly, cell proliferation continues posteriorly, allowing the elongation of the body axis (Bénazéraf & Pourquié, 2013). The number of body segments and the size of the PSM are controlled by two different factors (Gomez et al., 2008; Gomez and Pourquié, 2009; Bénazéraf & Pourquié, 2013). First, the regulation of the posterior proliferation of cells extending the body axis during the process of somite formation (a long body has more somites). Second, the rate of segmentation, which results in somites differing in size (small segments lead to more somites and large segments lead to fewer somites). Unfortunately, there has been very little research on variation in somitogenesis and its impact on the phenotype of adult fishes beyond studies

conducted on zebrafish, the major model fish species in biology (Lele & Krone, 1996; Mushtaq et al., 2013).

Astyanax mexicanus, the Mexican tetra, has become one of the major model systems in developmental biology, especially in evolutionary development (evo-devo), because of the extraordinary levels of phenotypic divergence that exist between surface and cave forms of the species (Jeffery, 2001; Borowsky, 2008; Hinaux et al., 2011). Previous research on lab-reared fish of the surface morph of *A. mexicanus* indicates that it exhibits significant phenotypic plasticity in body form and its axial skeleton in response to temperature changes during development. Reyes Corral and Aguirre (2019) found that fish reared at lower and higher temperatures had more vertebrae than fish reared at intermediate temperatures, and the ratio of precaudal to caudal vertebrae declined with temperature (Fig. 5). Body shape was also impacted by temperature variation and covaried with vertebral number, with fish having more vertebrae generally being more streamlined than those with less vertebrae. When these differences develop is not clear since the fish in the study were several months old and already exhibited differences by the time data collection began.

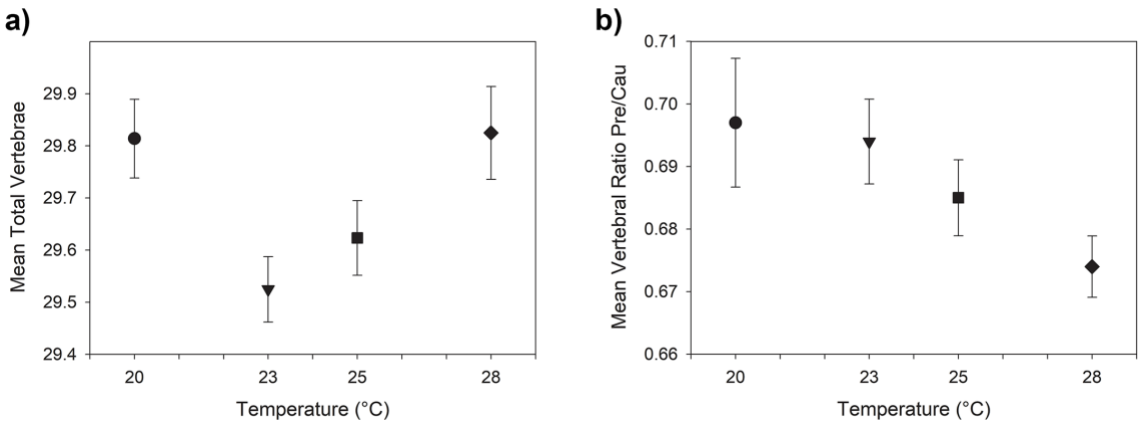

Figure 5: Vertebral number variation and the ratio of precaudal to caudal vertebrae in lab reared specimens of *Astyanax mexicanus* from Reyes Corral and Aguirre (2019).

The goal of this study is to use lab-reared *A. mexicanus* surface fish to examine whether temperature variation impacts the embryonic development of the body by measuring somite number, body length, the unsegmented tail length, and somite length in fish embryos reared at different temperatures. The rate of somitogenesis and general body growth slows down significantly at low temperature treatments and speeds up at high temperature treatments (Schröter et al., 2008). It is not generally known whether other aspects of body axis formation in fishes like somite size, embryonic body length, or unsegmented tail length are affected by temperature variation during early embryonic development once changes in general developmental rate are accounted for. There have not been studies examining the impact of temperature on somite formation in characids despite the great variation in body form and vertebral number that this family exhibits and its ecological important in Neotropical ecosystems (Mirande, 2010; Oliveira et al., 2011; Pereira et al., 2011; Escobar-Camacho et al., 2015). This study will give us a better understanding of how temperature influences the early stages of the development of the body axis in this emerging model species. It will also provide a baseline for future studies

examining the influence of phenotypic plasticity on body form variation in characids colonizing new habitats.

Methods

Fish Maintenance and Breeding

Procedures for this study were approved by the Institutional Animal Care and Use Committee (IACUC) of DePaul University (DePaul IACUC Protocol# 2018-002). The fish used for this experiment are from a breeding colony of the surface form of *A. mexicanus* held in the Research Support Facility of DePaul University since 2014. The founders of the colony came from the Jeffery Laboratory at the University of Maryland and constituted a 4th generation of lab-raised fish originally collected from the Rio Grande drainage basin. *Astyanax mexicanus* in the DePaul breeding colony are maintained on a 14:10 light dark cycle at 22°C in 5-20 gallon tanks. As part of standard care procedures, approximately 33% water changes are conducted twice a week and water quality is monitored weekly. Their ordinary diet consists of standard TetraMin Tropical Flakes administered daily.

Crossing

Adult *A. mexicanus* between one and four years old were used to set up the genetic crosses (Fig. 6). Prior to setting up the cross, female breeders were fed daily double rations of food including one ration of high protein freeze-dried blood worms for two weeks to stimulate reproductive condition. Tanks were prepared to set up four

crosses (Cross 3: summer 2017 and Crosses 14, 15, and 18: summer 2018). Of these, three were used in this experiment because the preservation of the embryos from the first cross performed (Cross 3) was not adequate for detailed anatomical analysis. Aquaria were rinsed several times, filled with clean water, and aerated using an air bubbler attached to a sponge filter. On the day of the cross, one male and one female were transferred approximately at 8:00 am to an aquarium with clean water and the temperature of the aquarium was raised 3-4°C (25°C) above the colony's normal temperature to induce mating (Borowsky, 2008). After the room lights turned off, females spawned approximately 300 to 500 eggs (possibly more) into the water column that night, typically beginning between 9 pm and 12 am. Tanks were checked for eggs approximately every 30 minutes after the room lights turned off using a red light. Once observed, eggs were collected from the bottom of the tank using a thin suction hose and distributed into petri dishes in incubators set to 20°C (100 eggs), 24°C (100 eggs), and 28°C (100 eggs) (Borowsky, 2008). Since 100 eggs are a large number for one petri dish, these were split into two petri dishes per temperature treatment, such that each had 50 eggs. Three water tanks were set to temperatures of 20°C, 24°C, and 28°C to serve as a water source of the correct temperature for water changes. The water of the petri dishes was changed three times a day, in the morning, afternoon, and at night.

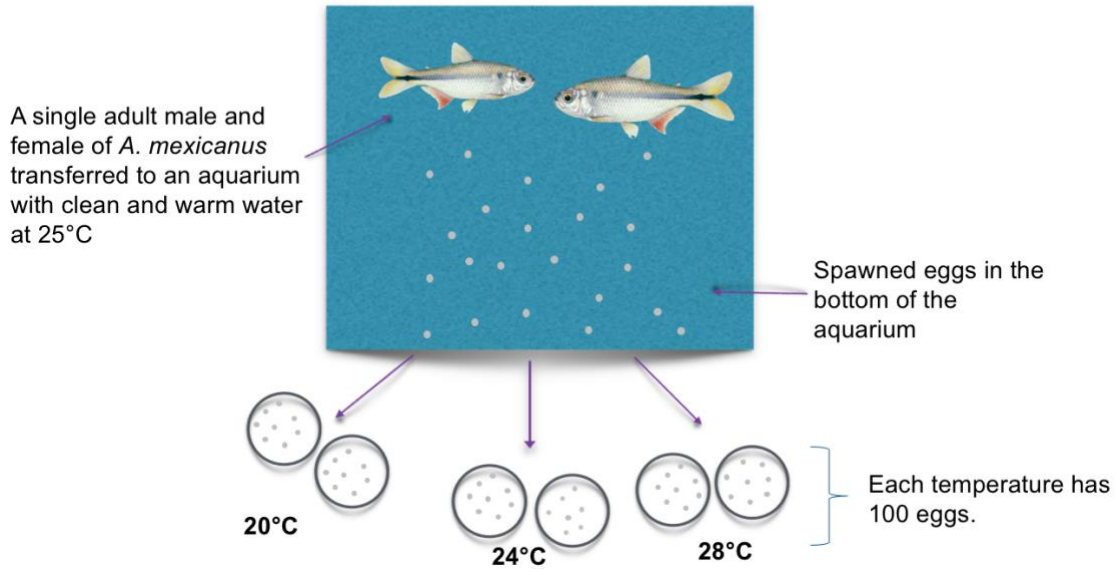

Figure 6: Setting up the genetic crosses of *Astyanax mexicanus*. Illustration showing a male and female *A. mexicanus* in the aquarium, spawned eggs in the bottom of the tank, and petri dishes with eggs distributed in the three temperature treatments. *Astyanax mexicanus* picture was taken from **http://www.fishesoftexas.org/taxa/astyanax-mexicanus.**

Specimen Preparation

Specimen preparation followed the method of Choi et al. (2018). Approximately three embryos with their chorion were collected every hour from each temperature treatment for each cross. Collecting embryos varied among the different temperatures. Embryos reared at 28°C and 24°C were collected beginning at three hours post fertilization (HPF) because of the high development rate of fish reared at high temperatures. Embryos reared at 20°C were collected beginning at five HPF because of the slower development rate of fish reared at low temperatures. Collected embryos were fixed overnight in 4% paraformaldehyde and preserved at 4°C. After collecting all samples, embryos were dechorionated using a pair of fine tweezers under the dissecting

microscope. Removing the chorion from very young embryos is much harder than removing it from older embryos because the chorion membrane gets softer as the embryos develop. Consequently, more young embryos were damaged during this process than older embryos. In order to prevent contamination before removing the chorion membrane, the bench, dissecting microscope, tweezers, and petri dishes were treated with MBP RNase-free spray (VWR). Dechorionated embryos were washed several times with 1X PBS to stop fixation and then dehydrated in a series of 100% methanol (MeOH) washes with 1 mL each at room temperature (100% MeOH 4 times for 10 min and 100% MeOH once for 50 min). Once dehydrated, embryos were stored at -20° C for future use.

Mounting Specimens

To begin collecting the data from the embryos, specimens were rehydrated with a series of washes of 100% methanol and 1X PBST (3:1, 1:1, 1:3,100% 1X PBST, for five minutes each). Rehydrated embryos were stained with 5µl of DAPI in 995µl of 1X PBS for thirty minutes in the dark at room temperature and washed twice with 1X PBS at room temperature for five minutes. DAPI is a fluorescent blue stain that binds to adenine and thymine rich regions in the nucleus of cells. DAPI is useful for highlighting embryonic structure and makes it easier to analyze images taken of embryos. Embryos were placed in a petri dish and the yolk of embryos that were 10 HPF and older was gently removed using fine tweezers under the dissecting microscope to facilitate positioning of the embryos on the slides. Removing the yolk was easier for older embryos because their yolk gets softer as they develop and their body elongates which give enough space to remove it. All materials were cleaned using RNase-free disinfectant

spray from Molecular Bio Products (MβP) to avoid any contamination during the process of removing yolk. A single cavity well microscope slide (Fisher Scientific) was placed on a light microscope, a few drops of 1X PBS were added to the well, and the embryo was transferred to the well using transfer pipette. Using 1X PBS instead of mounting medium made the mounting process much easier. Embryos were gently positioned on their lateral side using a 10µl pipette tip and a square cover slip was added. The slide was then left to dry in the dark for 10 minutes at room temperature and then taken to the confocal microscope for imaging.

Visualization

A Leica SPE/DMI 4000 confocal microscope was used to image embryos using the 488 nm laser for DAPI. The slide with the mounted embryo at intermediate and later HPF was placed upside down on the stage of the confocal microscope, but earlier HPF were not mounted or placed upside down on the stage because of their yolk. The embryo was focused in the center, the gain and the offset were adjusted to optimize visualization of each embryo. The format of the pictures was set to XY: 2048 * 2048, the speed to between 400 and 600 Hz, the acquisition mode as XYZ, and the zoom as 1. The z-stack option was used to take stacks of images from the lowest to the highest points of the embryo. The number of images used for the z-stack differed among embryos because of the differences in developmental stage and size, which resulted in some embryos being thicker and requiring more images. For larger, more developed embryos that did not fit within a single horizontal frame of the microscope image, the tile scan option was used, which generates multiple horizontal frames that can be combined to give a single image.

The z-stack pictures were combined to generate one final 3D picture. A scale bar was added to each image by selecting the scale bar option on the confocal microscope and images were saved as tifs for measurement and analysis. Pictures were edited using Gimp version 2.10.12 and Photoshop CS6 to erase the yolk tissue left after removing the yolk.

Scoring of Major Developmental Landmarks and Measurements

To compare the development of embryos reared at different temperatures, the first appearance of several key developmental landmarks was scored following Hinaux et al., (2011) (Fig. 7). These include the eye, otic vesicle, the appearance of the first somite, 10 somites, 20 somites, 30 somites and 40 somites, the formation of the gut, and hatching. The HPF for embryos with a particular number of somites refers to the first appearance of an individual with that number of somites in a particular temperature treatment. Hatching was recorded when embryos released themselves from the chorion in the petri dish.

The following counts and measurements were also taken from each embryo using Image J version 1.51S (Fig. 8). Somite number was counted, with only somites that were completely formed (border lines visible) included in the counts (Fig. 8A). To facilitate counting, the multi-point tool was used, which allowed me to number each somite (segment) to reduce the likelihood of counting errors. Body length (BL) was measured from the tip of the snout to the end of the tail along the main axis of the embryos using segmented lines to account for the bending of the body (Fig. 8B). The length of each somite was measured along the anterior posterior axis of the body from the anterior most point to the posterior most point of each somite (Fig. 8C). The point tool was used to make a straight line in the middle of the somite following the method of Schröter et al.

(2008). Finally, unsegmented tail length (UTL) was measured using a straight line or a segmented line from the posterior end of the last differentiated somite to the posterior end of the tail (Fig. 8D).

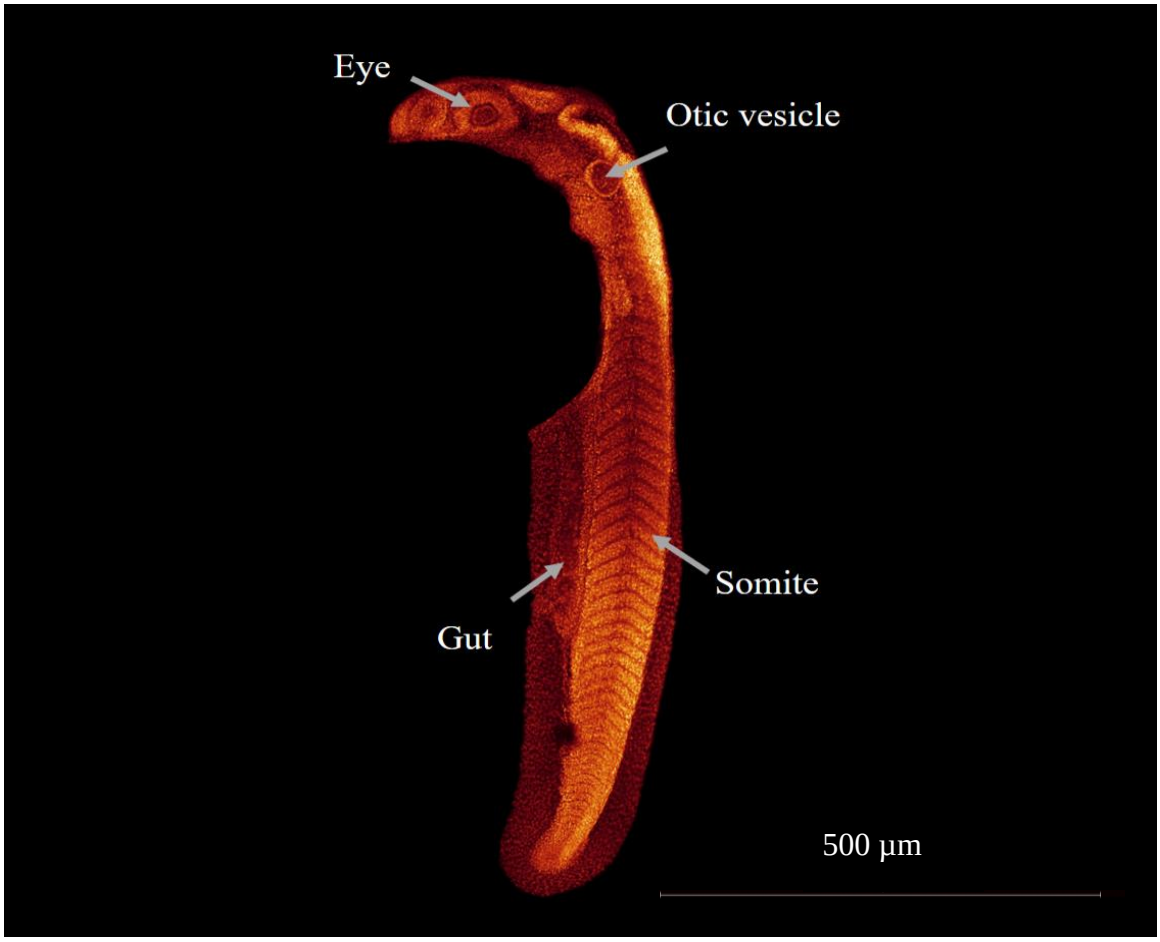

Figure 7: *Astyanax mexicanus* embryo from cross 15 reared at 28°C-20 HPF. The picture shows some major developmental landmarks in *A. mexicanus* such as the eye, otic vesicle, somites, and gut. The yolk has been removed from the image to facilitate mounting of the specimen on the slide.

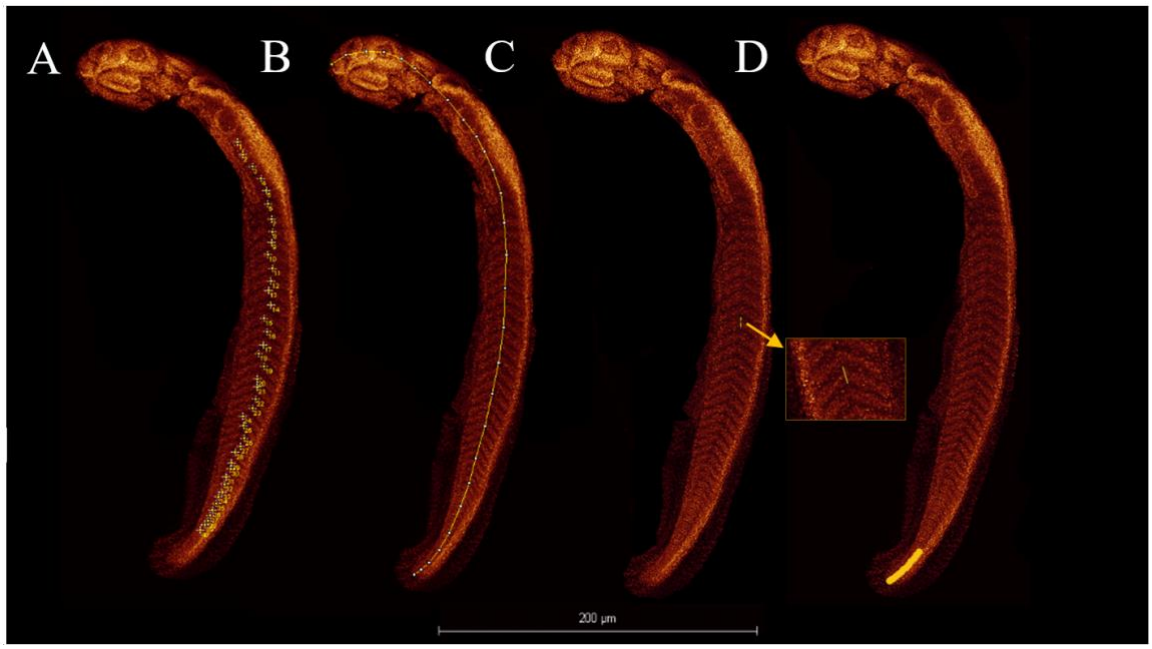

Figure 8: Measurements taken from embryos using Image J. Specimen pictured is a 34 hpf embryo from cross 14 reared at 20°C. **(A)** Somites were counted by adding numbers from the first to the last somite. **(B)** Body length was measured using segmented lines from the tip of the snout to the end of the tail. **(C)** Somite length was measured using a straight line from the anterior most point to the posterior most point in the middle of each somite. **(D)** Unsegmented tail length was measured by drawing a segmented line from the last differentiated somite to the end of the tail. All measures were taken in microns.

Statistical Analysis

To examine how the temperature treatment affected the development of body length, somite number, and unsegmented tail length, I conducted three ANCOVAs in R version 3.5.0 (The R project for Statistical Computing). ANCOVA differs from ANOVA in allowing the inclusion of a continuous covariate. The linear models were fit with body length (in microns, BL), somite number (SN), and unsegmented tail length (in microns, UTL) as the response variables, hours post fertilization (HPF) as a covariate, and temperature (T) as the discrete independent variable or experimental treatment being tested. The full models including the interaction between temperature and HPF were evaluated first and a model simplification approach was employed to test the effect of

temperature if the interaction was not statistically significant. The rates of growth in BL, SN, and UTL were estimated for each temperature treatment as the slopes of the regression of each of these response variables on HPF.

To examine if temperature impacts somite formation and the unsegmented tail length when differences in general developmental rate are accounted for, I conducted two additional ANCOVAs in R. Linear models were fit with somite number and unsegmented tail length as the response variables, body length as the covariate, and temperature as the experimental treatment. These analyses allowed me to test the impact of temperature on somite developmental rate and the UTL, controlling for the effect of temperature on general developmental rate as inferred by growth in body length. That is, does temperature have an effect on somite formation or UTL in fish grown at different temperatures that are at the same developmental stage? I ran the same analyses with somite number as the covariate instead of body length, and body length and UTL as the response variables to see if standardizing by body length or somite number made a difference. The result was the same (Appendix S2), so only the first analysis is presented in the Results section.

To explore the relationships among variables and the combined effects of multiple variables on SN, BL, and UTL, three multiple linear regression analyses were conducted in R version 3.4.4. Multiple linear regression is used to model the effect of multiple continuous explanatory variables on a response variable. The predictor variables included somite number, body length, unsegmented tail length; hours post fertilization, and temperature, depending on the analyses. Backward stepwise regression was used to

eliminate the independent variables one at a time starting with that with the largest P-value (least significant) in the model.

Finally, the effect of temperature on the size of the somites over development was analyzed in R version 3.6.1. I examined how somite size varied both within and across temperature treatments (20ºC, 24ºC, and 28ºC). The length of every somite was measured in microns for each embryo in ImageJ version 1.51S. To facilitate interpretation, specimens were subdivided into four developmental groups based on their total somite number: 10, 20, 30, or 40 somites. To increase sample sizes, specimens with 8-12 somites were included in the 10 somite group, 18-22 in the 20 somite group, 28-32 in the 30 somite group, and 38-41 in the 40 somite group. Given the rapid rate of development in *A. mexicanus*, these slight differences in somite number within the developmental groups were considered likely unimportant. For the within temperature treatment comparison, three plots were created, one for each temperature, showing how somite length varied along the body axis of individuals and at a particular somite across developmental stages as specimens developed. Somites are added posteriorly as embryos develop (Kimmel et al., 1995) until reaching the final number of 40 in *A. mexicanus*, so once established, they can be considered homologous based on their position in comparisons across specimens. For the among temperature treatment comparison, four plots were created, one for each developmental group (10, 20, 30, and 40 somite groups), showing how somite length differed along the body axis between temperature treatments as embryos developed. Unfortunately, there were no embryos in the 10 somite stage for the 28ºC treatment. The earliest embryos collected did not have differentiated somites and the first embryos to

show somites already had more than 12 somites. This is likely due to the extremely fast rate of development of *A. mexicanus* embryos at 28°C.

The R package "dplyr" was used to compute subgroup statistics by temperature, somite number, and developmental stage (10, 20, 30, and 40 somites). The mean length of each somite within a group, the standard deviation, and the number of specimens used to compute the means were calculated using the *summarize* function, and were used to compute the standard error of the mean (standard deviation divided by the square root of n), which in turn was used to generate error bars. I also used the *filter* function to separate the average mean length of each somite by the three temperature treatments, 20ºC, 24ºC, and 28ºC for the within temperature comparisons. The "ggplot2" package was used to create the plots.

Results

Development of *Astyanax mexicanus* Embryos

Astyanax mexicanus embryos develop as rapidly or more rapidly than zebrafish and are transparent like zebrafish embryos (Hinaux et al., 2011), which facilitates studying body axis formation during development. The formation of the body axis starts early in development but varies across temperature treatments. Embryos develop at a higher rate at warmer temperature treatments compared to cold temperature treatments. The appearance of major anatomical landmarks in *A. mexicanus* embryos reared at different temperatures is summarized in Table 1.

Table 1: First Appearance of Major Anatomical Landmarks in *A. mexicanus* Embryos Reared at Different Temperatures. *The HPF for the 10 somites category refers to the first appearance of an individual with at least 10 somites in a particular temperature treatment and so on for the 20, 30, and 40 somites categories. Ranges of HPF indicate variation among crosses. Eye formation, otic vesicle and gut were counted at their first appearance in an embryo. Hatching was recorded when embryos released themselves from the chorion in the petri dish.*

Temperature Treatments			
Anatomical Landmarks	**20°C**	**24°C**	**28°C**
First Somite	13-15 HPF	9-10 HPF	8 HPF
Eye Formation	14-16 HPF	10-11 HPF	8 HPF
10 Somites	17-18 HPF	10-11 HPF	8 HPF
Otic Vesicle	17-18 HPF	11 HPF	8 HPF
20 Somites	20-22 HPF	13-15 HPF	10-11 HPF
Gut Visible	20-21 HPF	15-18 HPF	13 HPF
30 Somites	27-28 HPF	17- 19 HPF	14-16 HPF
40 Somites	32-33 HPF	23-24 HPF	18-19HPF
Hatching	36 HPF	24 HPF	20 HPF

Somitogenesis starts well before eggs hatch, with somites forming towards the middle of the embryonic body axis and continuing to form posteriorly until somite formation is complete. Body form changes as embryos develop, with the tail extending posteriorly, elongating the body. The anterior region of the body becomes compressed as the posterior region elongates, such that the first somites appear to move forward during

development (Fig. 9A). As somites continue forming along the axis and the body continue to elongate, the unsegmented tail length decreases (Fig. 9B). The timing and rate of somite formation varies across temperature treatments. At 28°C, somites start to form at 8 HPF and form very quickly, such that 10 or more somites are apparent shortly after the first somites become visible. At 24°C, somites start to from between 9 and 10 hours post fertilization, with 7 to 9 somites being visible at this stage. At 20°C, somites start to form between 13 and 15 hours post fertilization, ranging between 5 to 8 in number when they first become visible. Somites then continue to form at different rates depending on temperature until a total of 40 somites form, with the total number being the same across temperature treatments. Somite size varies along the axis, with somites generally decreasing in length as they develop posteriorly. Embryos hatch shortly after somitogenesis concludes, with embryos reared at 28°C beginning to hatch within 20 HPF, embryos reared at 24°C hatching within 24 HPF, and embryos reared at 20°C hatching within 36 HPF.

A)

B)

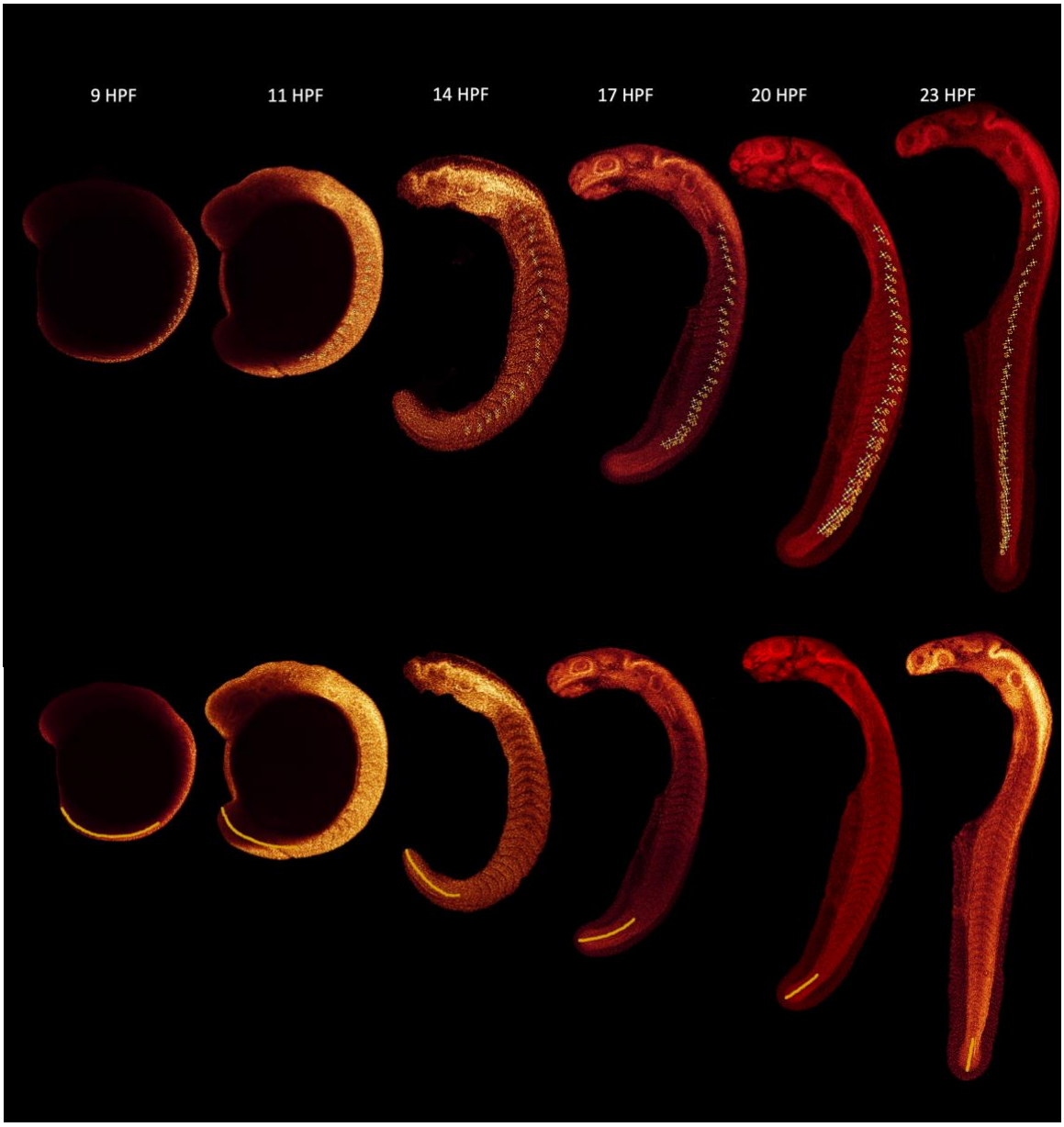

Figure 9: *Astyanax mexicanus* embryo from cross 14 reared at 24°C at different HPF. Pictures show the process of the body development and somite formation (A), and the reduction of the UTL from early to late development (B) (in microns).

Developmental Temperature Strongly Affects Body Growth, Somite Development, and Unsegmented Tail Length

Body length was strongly influenced by HPF, temperature, and the interaction of HPF and temperature indicating that temperature had a strong effect on the general developmental rate of *A. mexicanus* embryos (Table 2, Fig. 10A). Body length increased as embryos developed, however, the body grew slower at the colder temperature (20°C) than the two warmer temperatures (24°C and 28°C) (Fig. 11), such that the body length at 36 HPF of embryos reared at 20°C is similar in length to embryos that are 23 HPF and 20 HPF reared at 24°C and 28°C, respectively (Fig. 12). The rate of growth in body length, inferred from the slope of the natural log of body length regressed on HPF, was 0.0116 (microns/hour) at 20°C, 0.0155 (microns/hour) at 24°C and 0.0163 (microns/hour) at 28°C.

Multiple linear regression of the predictor variables on BL indicated that UTL, HPF, somite number, and temperature had significant effects on BL variation (Regression, df= 5,170, Fs= 87.06, P <0.001, R_2 = 0.7192), indicating that body length increases in embryos as somite number and HPF increase (Table 3).

Table 2: ANOVA table of the full ANCOVA model of body length. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (the test statistic), P value (Probability value), HPF (Hours Post fertilization), Temp (Temperature Treatments 20*°C*, 24*°C*, and 28*°C*), HPF:Temp (The interaction between hours post fertilization and temperature), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
HPF	1	0.87467	0.87467	366.1051	0.001
Temp	2	0.58129	0.29065	121.6538	0.001
HPF:Temp	2	0.03138	0.01569	6.5673	0.01
Residuals	207	0.49455	0.00239		

Table 3: ANOVA table of the reduced multiple regression model of body length. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (The test statistic), P value (Probability value), UTL (Unsegmented tail length), HPF (Hours post fertilization), SN (Somite number), Temp (Temperature Treatments 20°C, 24°C, and 28°C), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
UTL	1	12985886	12985886	296.3150	0.001
HPF	1	897052	897052	20.4692	0.001
SN	1	4868619	4868619	111.0933	0.001
Temp	2	326017	163008	3.7196	0.02624
Residuals	170	7450181	43825		

The development of somite number was also strongly influenced by temperature, hours post fertilization, and their interaction (Table 4). Somite number increased over embryonic development at different rates due to the effect of temperature on the general development rate. As expected, somites formed significantly faster at warmer temperatures than colder temperatures (Fig. 10B). The typical rate of somite formation (inferred from the slope of somite number regressed on HPF) was 1.5699 (somites/hour) at 20°C, 2.1459 (somites/hour) at 24°C, and 2.1878 (somites/hour) at 28°C. The greatest difference was between the two higher temperatures, 28°C and 24°C, relative to the 20°C temperature treatment.

Multiple linear regression of the predictor variables on SN indicated that BL, UTL, temperature, and HPF all significantly influenced somite number (F_s= 506.9, df= 5,170, $P < 0.001$, $R^2 = 0.937$). The model indicated that as BL and HPF increase and UTL decreases, somite number increases (Table 5).

Table 4: ANOVA table of the full ANCOVA model of somite number. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (The test statistic), and P value (Probability value), HPF (Hours post fertilization), Temp (Temperature Treatments 20*°C*, 24*°C*, and 28*°C*), HPF:Temp (The interaction between hours post fertilization and temperature), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
HPF	1	7127.9	7127.9	976.47	0.001
Temp	2	8292.5	4146.3	568.01	0.001
HPF:Temp	2	363.1	181.5	24.87	0.001
Residuals	176	1284.7	7.3		

Table 5: ANOVA table of the reduced multiple regression model of somite number. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (The test statistic), P value (Probability value), BL (Body length), UTL (Unsegmented tail length), Temp (Temperature Treatments 20*°C*, 24*°C*, and 28*°C*), HPF (Hours post fertilization), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
BL	1	11464.8	11464.8	1874.5291	0.001
UTL	1	3138.2	3138.2	513.1026	0.001
Temp	2	49.5	24.8	4.0487	0.01915
HPF	1	850.2	850.2	139.0050	0.001
Residuals	170	1039.7	6.1		

Unsegmented tail length decreased as embryos developed, the body became longer and the number of somites increased, with the rate of decrease being strongly influenced by temperature (Table 6). Unsegmented tail length declined significantly faster at warmer temperatures than colder temperatures (Fig. 10C). The typical rate of reduction of the unsegmented tail length was -0.0296 (microns/hour) at 20°C, -0.0403 (microns/hour) at 24°C, and -0.0448 (microns/hour) at 28°C (as inferred from the slope of the natural log of the unsegmented tail length regressed on HPF).

The multiple regression indicated that the factors influencing UTL included body length and somite number. This overall model was significant (Regression, df= 2,173,

Fs= 357.1, P <0.001, R_2 = 0.805), indicating that as body length and somite number increase, the unsegmented tail length decreases (Table 7). Temperature treatments, crosses, and hours post fertilization did not explain significant portions of the variation in UTL.

Table 6: ANOVA table of the full ANCOVA model of unsegmented tail length (UTL). *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (The test statistic), P value (Probability value), HPF (Hours post fertilization), Temp (Temperature Treatments 20°C, 24°C, amd 28°C), HPF:Temp (The interaction between hours post fertilization and temperature), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
HPF	1	3.00246	3.00246	457.226	0.001
Temp	2	2.75367	1.37683	209.669	0.001
HPF:Temp	2	0.17999	0.08999	13.704	0.001
Residuals	176	1.15574	0.00657		

Table 7: ANOVA table of the reduced multiple regression model of UTL. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (The test statistic), and P value (Probability value), HPF (Hours post fertilization), HPF:Temp (The interaction between hours post fertilization and temperature 20°C, 24°C, and 28°C), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
HPF	1	3.0025	3.00246	452.61	0.001
HPF:Temp	2	2.9086	1.45430	219.23	0.001
Residuals	178	1.1808	0.00663		

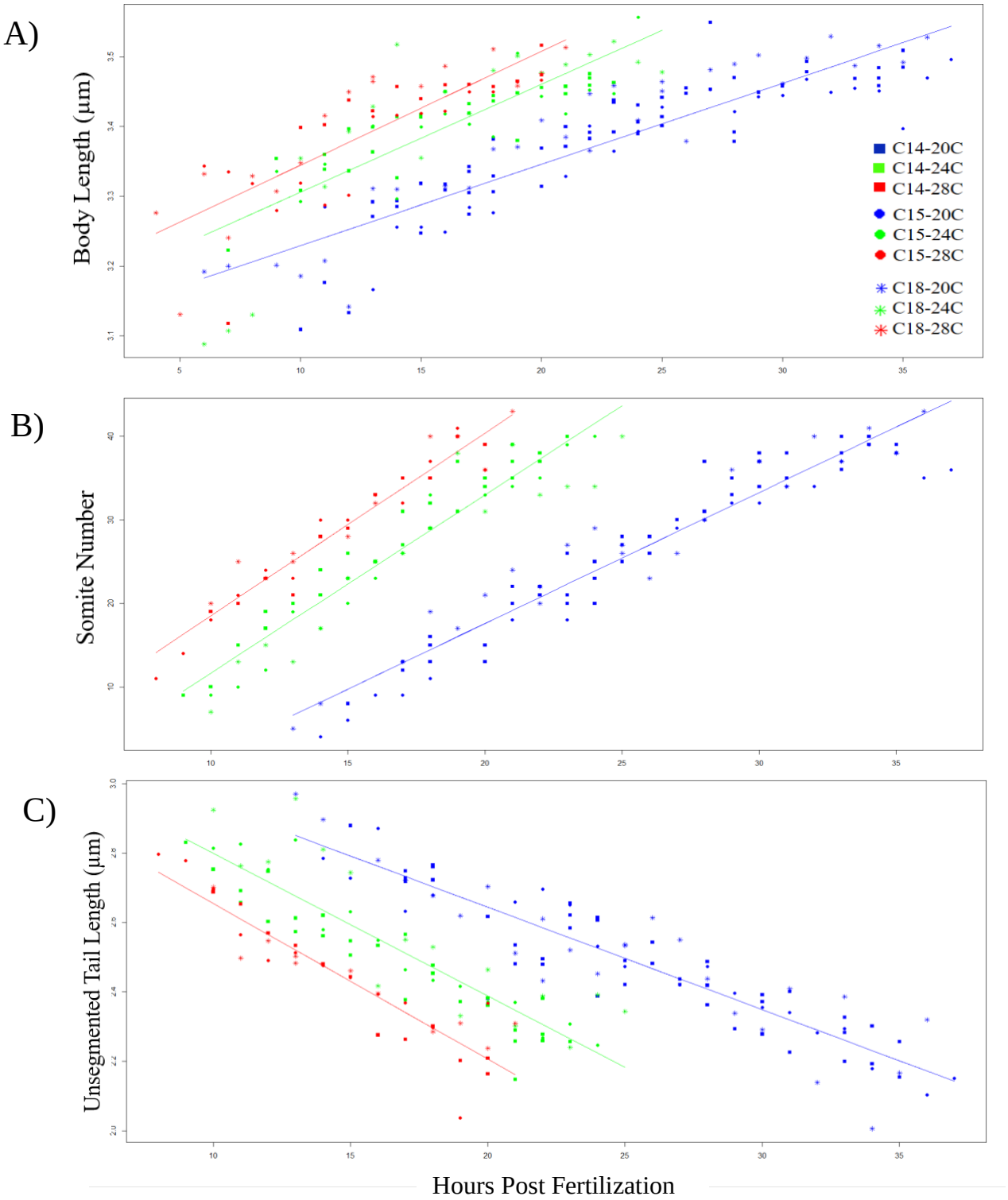

Figure 10: Development of body length, somite number, and unsegmented tail length in embryos of *Astyanax mexicanus* subjected to 20°C, 24°C, and 28°C temperature treatments. Points are individual embryos coded by temperature (color) and cross (symbol). (A) Body length (in microns), (B) somite number, and (C) unsegmented tail length (in microns) plotted as a function of hours post fertilization.

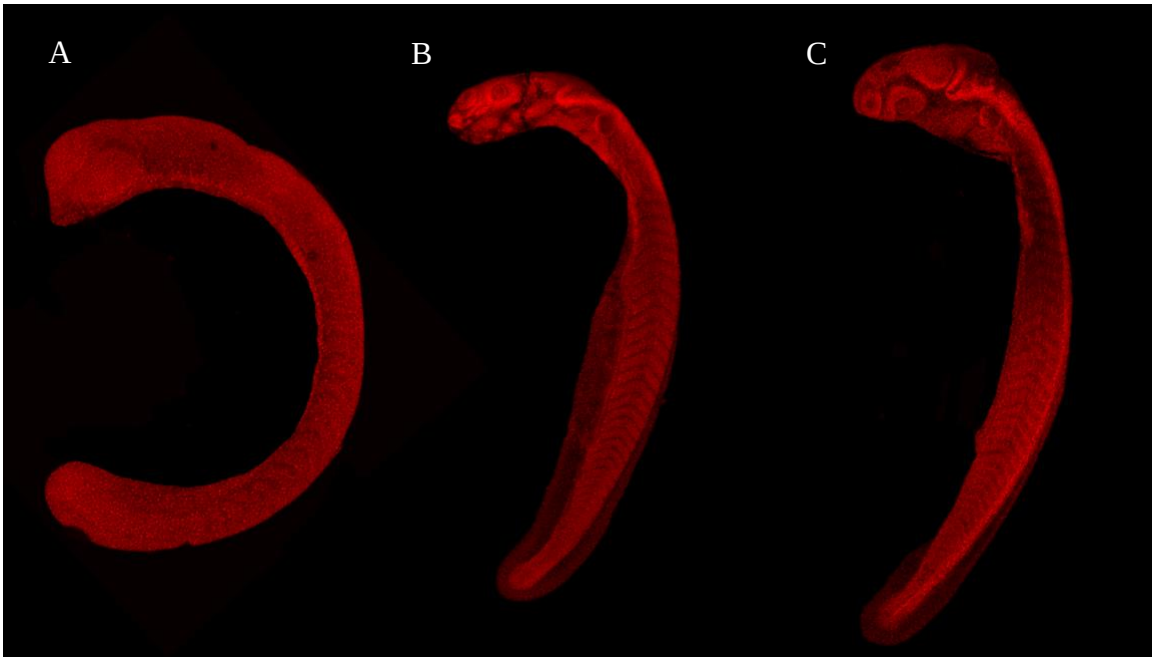

Figure 11: Differences in the general developmental rate of *A. mexicanus* embryos from Cross 14 all shown at 20 HPF but reared at different temperatures. (A) 20°C, (B) 24°C, and (C) 28°C. Yolk sac removed to facilitate visualization.

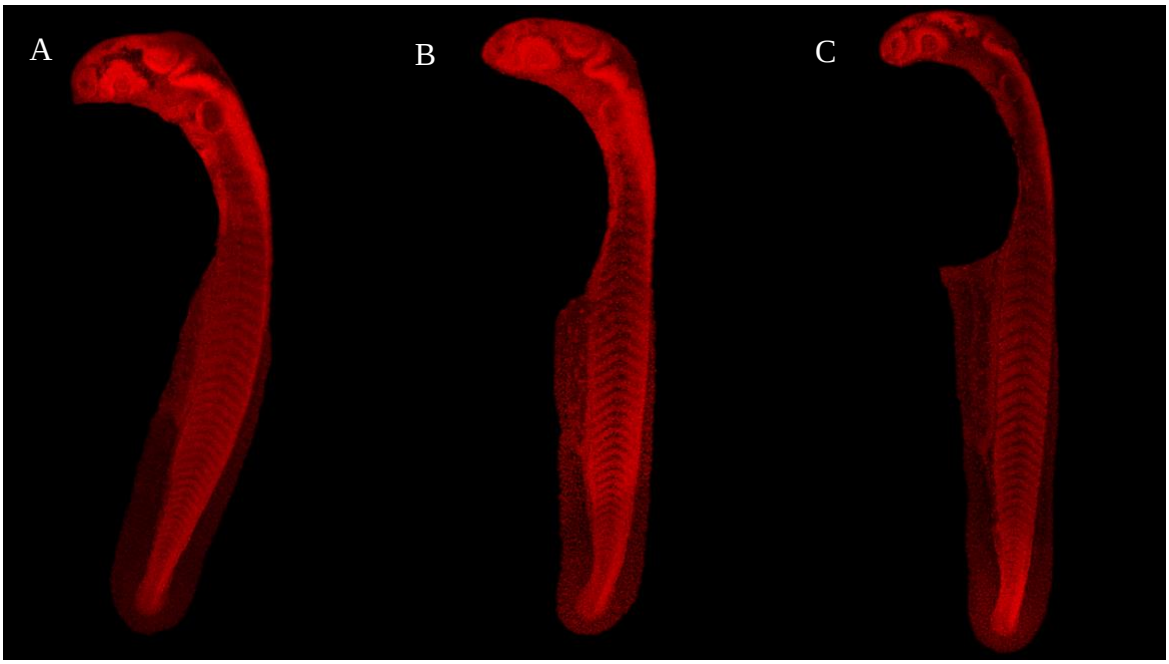

Figure 12: Embryos from Cross 15 with similar body lengths but reared at different temperatures. (A) 20C-36 HPF, (B) 24C-23 HPF, and (C) 28C-20 HPF. Yolk sac removed to facilitate visualization.

Does Temperature Impact Somite Formation and Unsegmented Tail Length When Accounting for Differences in General Developmental Rate?

To examine whether temperature impacts somite formation and unsegmented tail length once its general effect on developmental rate is accounted for, I conducted two ANCOVAs with temperature as the experimental treatment, the natural log of body length as the covariate and somite number and the natural log of the unsegmented tail length as the response variables. This allowed me to examine whether the number of somites and the unsegmented tail length differed between embryos of the same length grown at different temperatures.

The full ANCOVA model for somite number was not significant (Table 8), so the temperature and the interaction of body length and temperature were removed from the model by using a backwards stepwise regression. The impact of body length on somite number was significant (Table 9). As expected, somite number increases when the body length elongates, but temperature has no effect on changing the rate of somite formation when variation in body length was accounted for.

Table 8: ANOVA table of the full ANCOVA model of somite number. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (test statistic), P value (Probability value), BL (Body length), Temp (Temperature Treatments 20°C, 24°C, and 28°C), BL:Temp (The interaction between body length and temperature), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
BL	1	12214.1	12214.1	490.2841	0.001
Temp	2	41.0	20.5	0.8221	0.4413
BL:Temp	2	80.1	40.0	1.6075	0.2034
Residuals	169	4210.2	24.9		

Table 9: ANOVA table of the reduced model of somite number. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (test value), P value (Probability value), BL (Body length), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
BL	1	12214.1	12214	487.86	0.001
Residuals	173	4331.2	25		

The full ANCOVA model for UTL showed that the interaction between body length and temperature was not significant but temperature was marginally significant, even when developmental rate was standardized by including body length as a covariate (Table 10). A reduced model in which the interaction was removed showed that temperature remained significant (Table 11). Unsegmented tail length was slightly longer in fish reared at 24°C than fish of the same body length reared at 20°C and 28°C, despite the fact that total somite number was the same between temperature treatments. This indicates that the proportion of the body covered by somites is greater in the 20°C and 28°C temperature treatments than in the 24°C treatment, suggesting that developmental temperature can affect somite size. Similar results were obtained when standardizing by somite number instead of body length (Appendix S2).

Table 10: ANOVA Table of the full model for UTL. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean of the sum of squares), F value (F statistics), and P value (Probability value), BL (Body length), Temp (Temperature Treatments 20°C, 24°C, amd 28°C), BL:Temp (The interaction between body length and temperature), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
BL	1	3.7092	3.7092	209.9340	0.001
Temp	2	0.1081	0.0540	3.0577	0.04956
BL:Temp	2	0.0029	0.0015	0.0834	0.91998
Residuals	172	3.0389	0.0177		

Table 11: ANOVA Table of the reduced model for UTL. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean of the sum of squares), F value (F statistics), and P value (Probability value), BL (Body length) and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
BL	1	3.7092	3.7092	212.1692	0.001
Temp	2	0.1081	0.0540	3.0903	0.04799
Residuals	174	3.0419	0.0175		

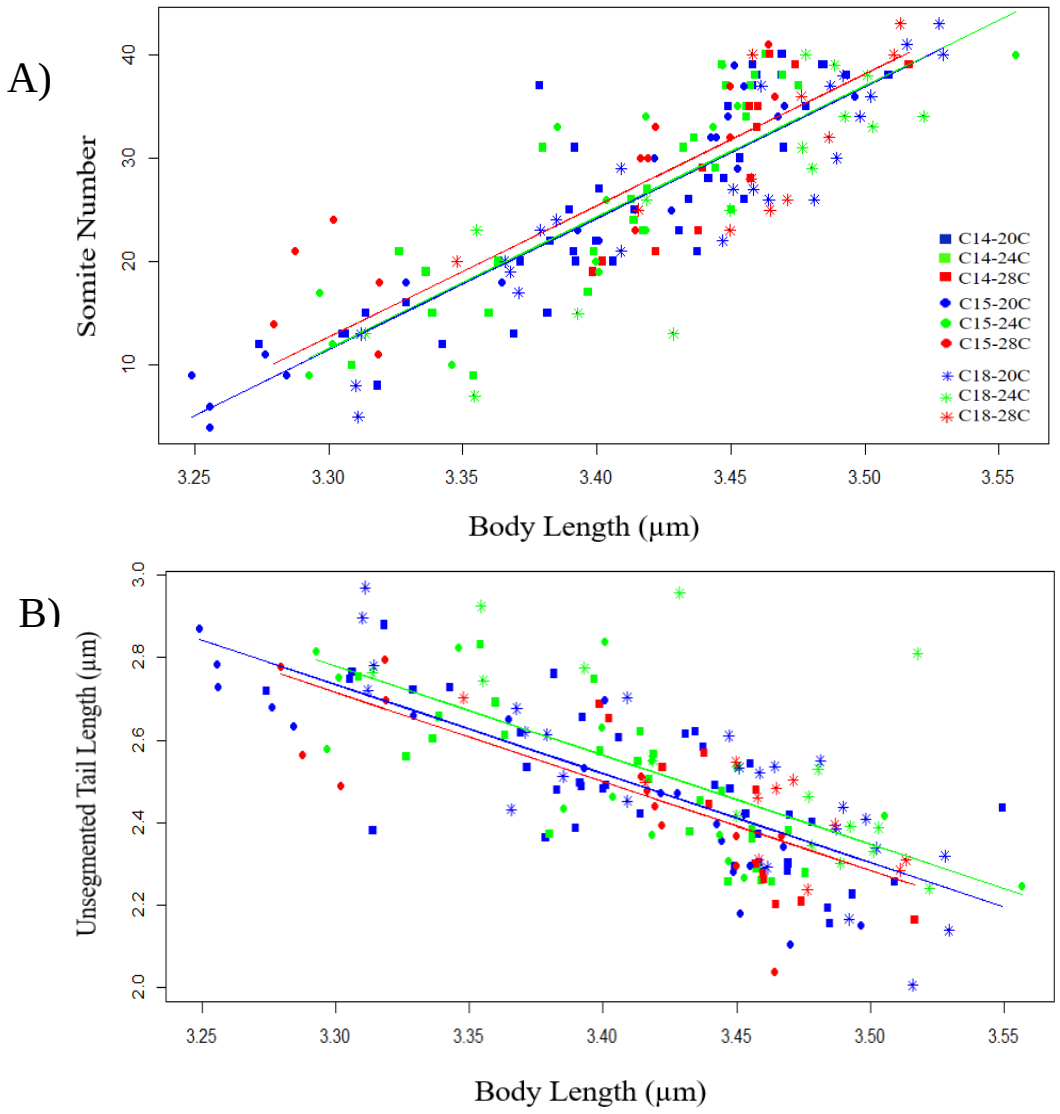

Figure 13: The effect of temperature on somite formation and unsegmented tail length when development rate is standardized using body length as a covariate in embryos subjected to 20°C, 24°C, and 28°C temperature treatments. (a) Somite number, (b) Unsegmented tail length (in microns) plotted as a function of body length (in microns).

Does Developmental Temperature Impact Somite Length?

We know that temperature influences the general rate of development, so to examine whether temperature affects somite length, comparisons between embryos reared at different developmental temperatures were standardized by somite number. Embryos were divided into groups with 8-12 somites (representing the 10 somite group), 18-22 (20), 28-32 (30), and 38-41 (40) somites. The length of each somite across crosses (C14, C15, and C18) of the same group (e.g., 8-12 somites) were analyzed by calculating the mean somite length (± standard error of the mean) and comparing across temperature treatments or developmental stages.

First, I examined how somite length changed over time and along the body axis to understand their developmental pattern at each temperature. For the 20ºC treatment (Fig. 14A), the anterior somites that develop first grow in length over development as new somites are forming posteriorly through the 20 and 30 somite stages. The length of the anterior somites at the 30 somite stage is similar to the length they have at the 40 somite stage. Between the 20 and 30 somite stages, there is a relatively large difference in somite length in the anterior somites but this disappears posteriorly (beginning at about the 15th somite). For the posterior somites, there is a substantial difference in somite length between the 40 somite stage and the 20 and 30 somite stages, indicating significant growth over development in the posterior somites. As new somites form posteriorly, however, they have progressively shorter lengths. Combined with the increase in length of the anterior somites over development, the effect is a large difference in somite size along the body axis such that at the final 40 somite stage, the anterior somites are almost three times the length of the posterior somites (Fig. 14A). For the 24ºC and 28ºC

treatments, much of the variation in somite length along the axis and over development is similar to the 20ºC treatment, so I focus on describing the differences.

For the 24ºC treatment, the difference in length in the anterior somites was not as great between the 10 and 20-30 somite stages as seen in the 20ºC treatment. The length of the anterior somites in the 40 somite stage seemed more divergent from those of the earlier stages indicating substantial growth in these somites. Somite lengths were generally similar between the 20 and 30 somite stages along the body axes. Posteriorly, again it is the 40 somite stage that appears distinct from the younger stages with longer somites. The overall pattern of decline in somite length is present as it was for the 20ºC treatment, appearing perhaps even more exaggerated (Fig. 14B).

For the 28ºC treatment, there was noticeably less divergence in somite length between the developmental stages that were sampled. The 40 somite stage appeared to have somewhat longer anterior somites relative to the 20 and 30 somite stage, but this difference largely disappeared in the posterior somites, which appeared similar in length between the 40 somite stage and the 20 and 30 somite stages. That is, somites located more posteriorly (somites 20-30) seemed to grow little between the 30 and 40 somite stages, unlike the pattern seen at 20 and 24ºC. The overall decline in somite length from the anterior to the posterior portion of the body was even more exaggerated than at 20 and 24ºC, with the posterior most somites appearing to be close to 1/6 the length of the longest anterior somites (Fig. 14C).

Second, I examined the effect of temperature on somite length by plotting average somite length across temperature treatments for each of the four developmental stages defined by somite number (8-12 somites for the 10 somite stage, 18-22 for the 20 somite

stage, 28-32 for the 30 somite stage, and 38-41 for the 40 somite stage). For the 10 somite stage, there was broad overlap in somite length with little difference between the 20 and 24ºC temperature treatments (Fig. 15A). By the 20 somite stage, there seemed to be more heterogeneity in somite length between the temperature treatments with embryos reared at 28ºC tending to have shorter somites and embryos reared at 24ºC tending to have longer somites than see in the other temperature treatments. These tendencies were not strong since there was substantial variation and the lines defining somite length along the body axis crossed between temperature treatments at different points (Fig. 15B). By the 30 somite stage, there still seemed to be some heterogeneity in somite length between temperature treatments in the anterior somites. The lengths of the anterior somites in the 28ºC temperature treatment tended to be lower and those in the 20ºC treatment tended to be higher than the other temperature treatments. However, by the 15th somite, there was little apparent difference in somite lengths across temperature treatments until the last differentiated somites (beginning around the 25th somite), when the temperature lines diverged slightly again (Fig. 15C). At the 40 somite stage, there was broad overlap and substantial heterogeneity in somite length for the anterior somites, but this changed posteriorly where there was clear differentiation between the 28ºC treatment and the 20 and 24ºC treatments beginning at about the 13th somite (Fig. 15D). Embryos reared at 28ºC had consistently shorter somites than embryos reared at lower temperatures. This is consistent with the limited growth seen in the posterior somites over development of embryos in the 28ºC treatment relative to embryos reared at lower temperatures (Fig. 14). Consequently, temperature did seem to have an effect on somite length during

embryogenesis in *A. mexicanus*, although the effect was relatively small and varied along the body axis and by developmental stage.

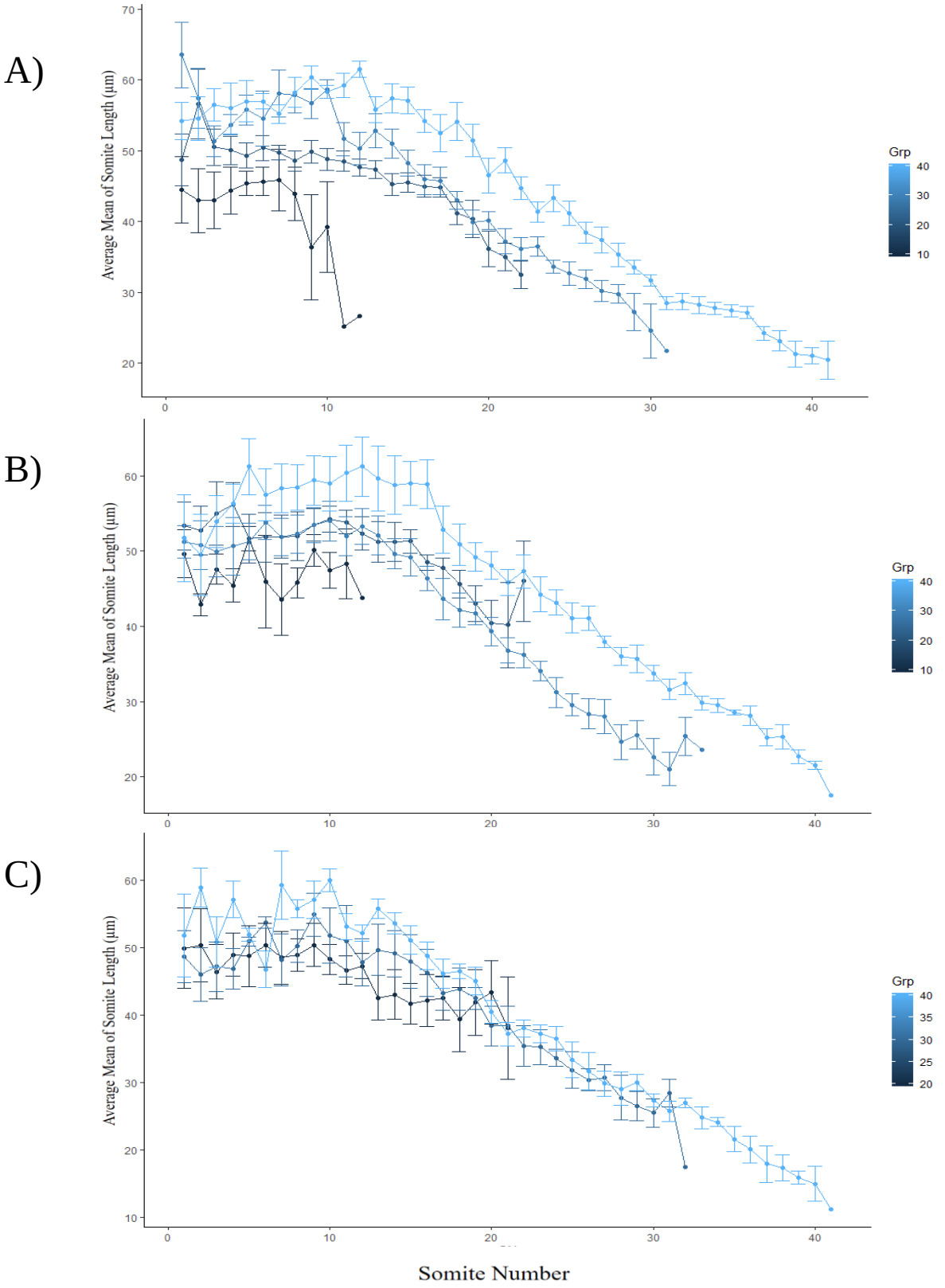

Figure 14: Somite Length Variation Between Developmental Stages at 20ºC, 24ºC, and 28ºC. The three graphs show average somite length (in microns ± standard error of the mean) plotted as a function of somite number. Embryos were divided into four groups based on somite number (8-12, 18-22, 28-32, 38-41 somites) at 20ºC (A), 24ºC (B), and 28ºC (C).

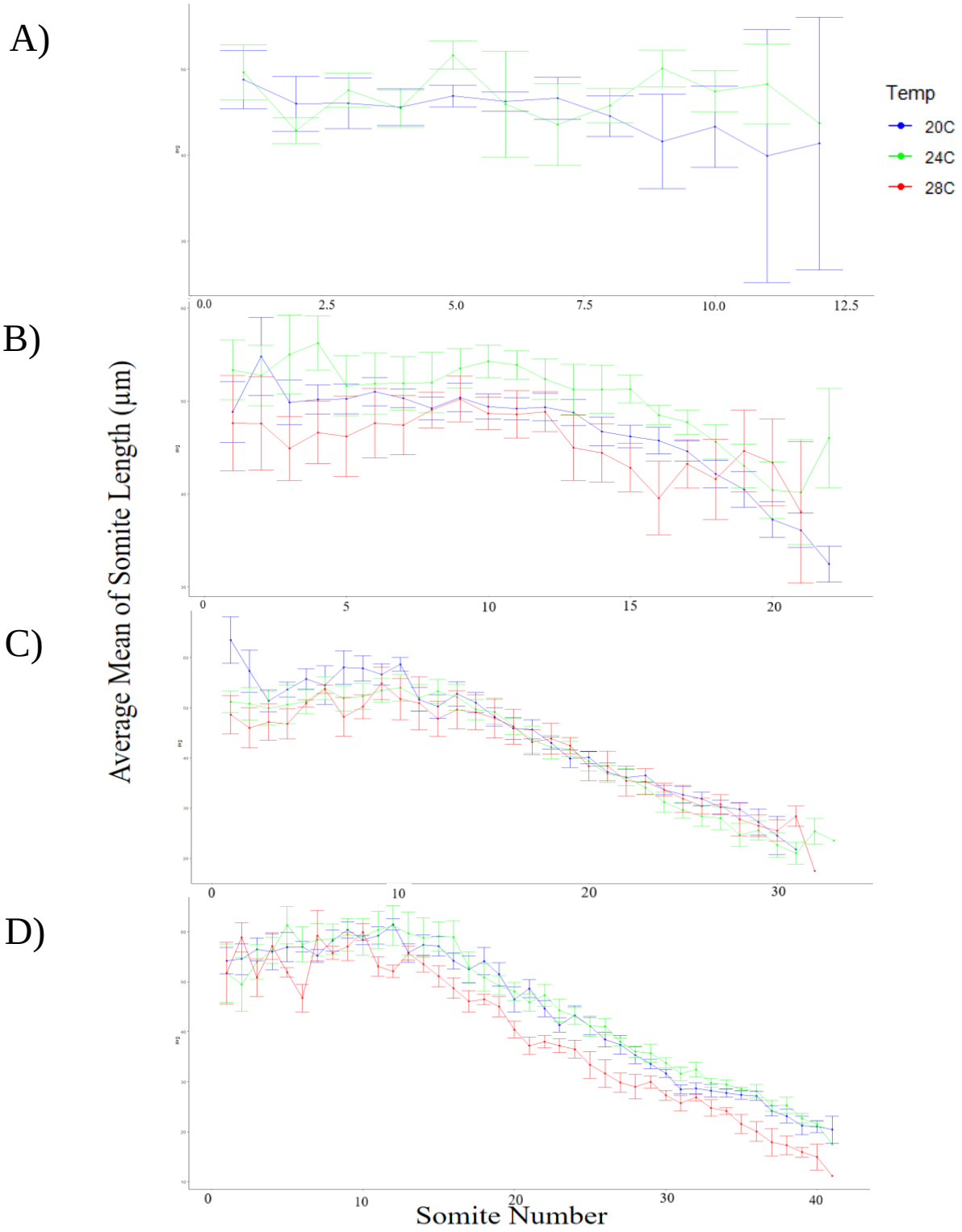

Figure 15: Somite Length Variation Between Temperature Treatments at Different Developmental Stages. The four graphs show the average somite length (in microns) as a function of somite number for each temperature treatment across crosses. Developmental stages were established based on somite number: 8-12 (A), 18-22 (B), 28-32(C), and 38-41(D) somites.

Discussion

The primary aim of this project was to study the impact of temperature fluctuations on the early stages of the development of the body axis in the model species *A. mexicanus*. Temperature is a critically important environmental factor known to impact many aspects of the general development, morphology, and physiology of ectotherms (Angilletta, 2009). Temperature has been shown to affect vertebral number in *A. mexicanus* (Reyes Corral & Aguirre, 2019) and many other fish species (McDowall, 2008). Since somite number and vertebral number are related (Fleming et al., 2015), it was hypothesized that temperature may also be impacting the formation of somites during embryogenesis. If so, I expected to see differences in somite number that coincide with differences in vertebral number documented in adults of this species. I also expected the general rate of body development to be higher in the warmer temperature treatments than colder temperature treatments based on previous research in zebrafish (Schröter et al., 2008) and *A. mexicanus* (Reyes Corral & Aguirre, 2019). I expected that somite size would not vary across temperature treatments, but that they would decrease in length posteriorly along the body axis.

Effects of Temperature on Body Growth

Temperature had a strong and significant impact on the developmental rate of *A. mexicanus* embryos. Embryos developed at a slower rate in colder temperatures and at a higher rate at warmer temperatures. At warmer temperatures, body length elongates posteriorly around 10 HPF for the 28°C treatment, 13 HPF for the 24°C treatment, and

around 20 HPF for the 20°C treatment (Fig. S1). The difference of body growth is very large for embryos reared at the 28°C and 20°C temperature treatments. Differences in developmental rate related to temperature have been well documented in fish species including zebrafish (Barrionuevo & Burggren, 1999; Delaunay et al., 2000; Schmidt & Starck, 2004; Hallare et al., 2005; Schröter et al., 2008). Higher temperatures are known to allow faster cellular activity, increasing metabolic rate (Clarke & Johnston, 1999; Deane & Woo, 2009). Temperature is also known to influence metabolic rate by increasing the production of growth hormone which induces body growth (Deane & Woo, 2009).

Whether changes in the rate of body development during embryogenesis at different temperatures lead to morphological changes in the body shape of adult fishes is unclear. However, temperature differences during early development can significantly impact the body shape of adults fishes (Sfakianakis et al., 2011) including that of *A. mexicanus* (Reyes Corral & Aguirre, 2019). In *A. mexicanus*, embryos reared at 20ºC tend to have more streamlined bodies than fish reared at 28ºC (Reyes Corral & Aguirre, 2019). Experiments that manipulate developmental rate during embryogenesis and then allow fry to grow at a constant temperatures to see whether differences in adult body shape are detectable could get at this issue.

Effects of Temperature on Somite Development

Somites obviously form over time as embryos develop (Schröter et al., 2008; Bajard et al., 2014; Jörg et al., 2015). Temperature affects the onset of somitogenesis, which starts earlier in warmer temperatures than colder temperatures. The rate of somite

formation also increases at warmer temperatures compared to colder temperatures. I expected that embryos reared at colder temperatures would form more somites and embryos reared at warmer temperatures would form less somites or that somite number would vary in a U-shaped pattern since the body growth is affected by temperature treatments and vertebral number varies in relation to temperature variation during development. For example, a study performed on *A. mexicanus* examined the number of vertebrae in fish reared at different temperatures (Reyes Corral & Aguirre, 2019). Mean total vertebral number showed a U-shaped pattern across temperature treatments, being high at the lowest and highest temperature treatments (20ºC and 28ºC) and low at intermediate temperatures (23ºC and 25ºC). This is a relatively common result for variation in vertebral number for fishes of the same species reared at different temperatures (Fowler, 1970).

Total somite number did not obviously differ across temperature treatments in my study, however. It seems that somitogenesis is synchronized with temperature variation and body growth despite the known impact of temperature variation on other serially repeating traits like vertebral number. This result was also observed in zebrafish, in which the number of total somites that form was not affected by changes in temperature during development (Schröter et al., 2008). Consequently, there appears to be a disconnect between the number of somites that form during early development and the number of vertebrae that form later. Thus, changes in the number of vertebrae seen in adult fish appear to be related to processes that happen after the somites differentiate.

Somite number and vertebrae number are related, but they are not exactly the same (Fleming et al., 2015). Somites differentiate into myotome and sclerotome. Each

sclerotome is divided into anterior and posterior parts. In general, a vertebra is formed from the posterior part of the sclerotome that is anterior to where it will develop and the anterior part of the sclerotome that is posterior to where it will develop (Fleming et al., 2015). Thus, it is not a single somite that forms a single vertebra. In zebrafish, the total number of somites is 30 (Schröter et al., 2008) and the total number of vertebrae is 33 (Bird & Mabee, 2003), indicating that there is a slight mismatch in how somites contribute to vertebral number relative to the accepted model (Fleming et al., 2015). In *A. mexicanus* the mismatch is much greater because somite number is 40 as seen in this study and in Hinaux et al. (2011), while the total number of vertebrae is 34 (Reyes Corral & Aguirre, 2019). This large mismatch in somite number relative to vertebral number indicates that the contribution of somites to vertebrae is likely much more flexible in *A. mexicanus* than in zebrafish or works in a different way. Future cell mapping experiments (Morin-Kensicki et al., 2002; Gomez et al., 2008) in *A. mexicanus* and other fish species in which somite and vertebral number show varying relationships may help decipher how general the relationship between somite number and vertebral number is.

Effects of Temperature on Unsegmented Tail Length

As somites begin to form in the anterior region of the body, there is an unsegmented region in the posterior part of the body. I measured this region and found that the unsegmented tail length (UTL) decreased as the somites formed despite the posterior elongation of the body through the growth from the tail bud cells (Bénazéraf & Pourquié, 2013). Controlling for developmental stage, the UTL did not differ between the extreme temperature treatments at 20°C and 28°C, but it did differ between these and the

24°C treatment. Embryos reared at 24°C had a slightly but significantly longer UTL than the 20 and 28°C treatments. Interestingly, adult *A. mexicanus* reared at intermediate temperatures of 23°C and 25°C had less vertebrae than fish reared at 20°C and 28°C (Reyes Corral & Aguirre, 2019). This might suggest a relationship between the UTL and the ultimate number of vertebrae that form. Unfortunately, there are multiple possible causes for why the UTL is longer in embryos reared at 24°C than embryos reared at 20°C and 28°C, and these may influence the process of segmentation in different ways. For example, 24°C embryos might develop shorter somites or they might have a longer body axis. The difference could also relate to the presomitic mesoderm (PSM) or the tail bud, both of which are within the UTL and not distinguishable phenotypically. These also could influence segmentation in different ways since the PSM provides the tissue for somite formation and the tail bud provides the cells for axis elongation (Dubrulle & Pourquié, 2004; Bénazéraf & Pourquié, 2013; Bénazéraf et al., 2017). However, the finding of a significant difference in the UTL caused by changes in temperature during development is a potentially important result that has not previously been documented in other fishes that I am aware of, including in zebrafish, and should be explored further in future studies.

Effects of Temperature on Somite Length

Somite size changes as embryos develop and according to their position along the body axis in *A. mexicanus* embryos reared at different temperature treatments. Somites begin to form anteriorly along the body axis and develop posteriorly (Kimmel et al.,

1995) until reaching their final number, which is approximately 40 in *A. mexicanus*. As new somites form posteriorly and the tail extends, the anterior somites grow in length. The new somites that form also begin at shorter lengths, resulting in a pattern of longer anterior and increasingly shorter posterior somites. This general pattern holds across temperature treatments although there seem to be differences among temperature treatments in the details. Somite length is not frequently measured in fishes, but in zebrafish, whose embryonic development has been studied in great detail, the first somites tend to be short, increasing in length after the first few, and then decreasing in length towards the tail, similar to the pattern I documented (Schröter et al., 2008; Jörg et al., 2015). Schröter et al. (2008) subjected zebrafish embryos to three temeprature treatments (21°C, 25°C, and 31°C) and measured somite length as they developed using timelapse movies. Somite length was smaller in the anterior somites, increased in length in the middle of the trunk, and decreased in length posteriorly as the embryos developed in all three temperature treatments. Simillar patterns have also been observed in mouse and chicken embyos (Tam, 1981), and may be quite general in vertebrates.

Interestingly, although vertebrae do not form from single somite, they also exhibit a pattern of longer anterior and shorter posterior vertebrae in many species (Bird & Mabee, 2003). In some cases, this pattern has been suggested to be adaptive (Aguirre et al., 2014; Ackerly & Ward, 2016). For example, Aguirre et al. (2014) found that more elongate limnetic threespine stickleback exhibited an increase in vertebral number in the caudal region of the body relative to oceanic and benthic stickleback populations, and suggested this may increase flexibility in the caudal region, which is important in burst swimming performance. It would be interesting to examine whether there is a general

correlation between the length of somites and vertebrae along the body axis because this may indicate that processes affecting the size of body segments very early in development may influence adaptively important traits like vertebral length in adult fishes.

There was also evidence of variation in somite length among embryos reared at different developmental temperatures. This variation was subtle and varied along the body axis and through development (Fig. 14). The most striking difference was that observed between embryos reared at 28°C and embryos reared at 20 and 24°C at the 40 somite stage. Embryos reared at 28°C seemed to have consistently shorter somites in the posterior region of the body than fish reared at 20 and 24°C. This was consistent with the lower growth rate of posterior somites between the 30 and 40 somite stage in embryos reared at 28°C. Embryos reared at this temperature develop extremely fast, completing somitogenesis at approximately 18 HPF and hatching at approximately 20 HPF. It seems possible that with this fast development comes the formation of somites that are shorter in length or have less time to grow in length than somites that form and grow more slowly at lower temperatures. Given the shorter somites, one might expect a shorter body length as well. Although body length did seem slightly shorter in embryos reared at 28°C relative to the 20 and 24°C embryos (Fig. S2), this difference was not statistically significant. Head length was not measured and could be accounting for some of the difference as well.

There is very little published on the effects of temperature variation on somite length in fishes. In zebrafish, Schröter et al., (2008) found that although somites developed slower at colder temperatures, somite length did not differ significantly

between homologous somites in embryos reared at different temperatures that were at the same developmental stage. Thus, the divergence in somite length documented here was unexpected. Replication of this study with a greater number of crosses is necessary to confirm the result obtained here. If repeatable, differences in somite size related to developmental temperature may contribute to the variation in vertebral number seen in adult fish that develop at different temperatures. Since the sclerotome of the somites forms the vertebrae, differences in somite size may affect the amount of tissue available for making vertebrae in different portions of the body axis, and thus influence the number and length of vertebrae that form along the body axis. More research on this topic is needed.

Limitations

There are limitations to my study that should be acknowledged for future research. One of the limitations is that embryos of *A. mexicanus* develop at a high rate at their normal developmental temperature of 24ºC and hatch within 24 HPF. The rate of development was even higher at the 28ºC temperature treatment. As a consequence, it was hard to collect embryos exhibiting all possible somite numbers because several somites formed within an hour at the warmer temperature treatments. I would suggest collecting embryos every 20 - 30 minutes in future studies to track the development of the body axis formation and to have a better understanding of somitogenesis rate in *A. mexicanus*.

Another limitation is all measurements of somite number; somite length, body length, and unsegmented tail length were taken on fixed and whole mounted embryos

which may affects the results. Embryos may shrink when placed in preservation solutions, which could distort some of the measurements taken. All embryos were preserved in the same way, so this should not have a substantial effect on the comparative analysis performed here. However, it may affect the value of the absolute measurements taken and also be problematic if my data are compared on measures taken from live specimens in other studies. Another study examined zebrafish development using time-lapse videos (Schröter et al., 2008). This allows for more a powerful analysis of somitogenesis because inter-individual noise is eliminated with this design, as are issues related to preservation artifacts. I would suggest using the same method to track the body axis development and somite formation in *A. mexicanus* embryos in future studies. In addition, one could track the same embryos as they develop into adults under the different temperature treatments to understand how variation in body axis development and somite length during embryogenesis relate to variation in body shape and vertebral number in adult fish. This will give us a better understanding of how variation in development during the earliest stages of body axis formation influence the phenotypic variation seen in adult fish.

Conclusions

The main conclusion of this study is that there is an influence of temperature on body growth, somite development, the unsegmented tail length, and somite length in lab-reared *A. mexicanus.* Temperature affected all variable measured through its known influence on general developmental rate. Surprisingly, temperature slightly influenced the UTL at 24ºC even when changes in general developmental rate were accounted for by standardizing both by body length and somite number. Embryos reared at 24°C had significantly longer UTL than embryos reared at 20 and 28°C. The significance of this temperature effect is unclear but given that adult *A. mexicanus* reared at similar temperatures of 23 and 25°C exhibited significantly less vertebrae than fish reared at other temperatures, it seems worthy of future research. The rate of body growth, somite development, and the unsegmented tail length differed among temperature treatments for all crosses. Somite length varies along the body axis decreasing posteriorly as seen in other fishes like zebrafish. Somite length varied among temperature treatments and seemed consistently smaller for the 40 somite developmental stage at 28ºC than at 20ºC and 24ºC. There have not been studies examining the impact of temperature on somite formation, body length, and the unsegmented tail length in characids despite the great variation in body form and vertebral number that this family exhibits and its ecological important in Neotropical ecosystems (Mirande, 2010; Oliveira et al., 2011; Pereira et al., 2011; Escobar-Camacho et al., 2015). Thus, this study provides a better understanding of how temperature influences the early stages of the development of the body axis in this emerging model species. It will also provide a baseline for future studies examining the

influence of phenotypic plasticity on body form variation in characids colonizing new habitats. Studying the impact of temperature on morphological variability gives us a better understanding of how environmental factors affect the phenotypic properties of species in nature and how these adapt to changes in their habitat. Future studies should examine how changes during embryogenesis relate to changes in adults and whether these changes are beneficial for their survival or not. Developing a mechanistic understanding of the patterns documented here will also be beneficial.

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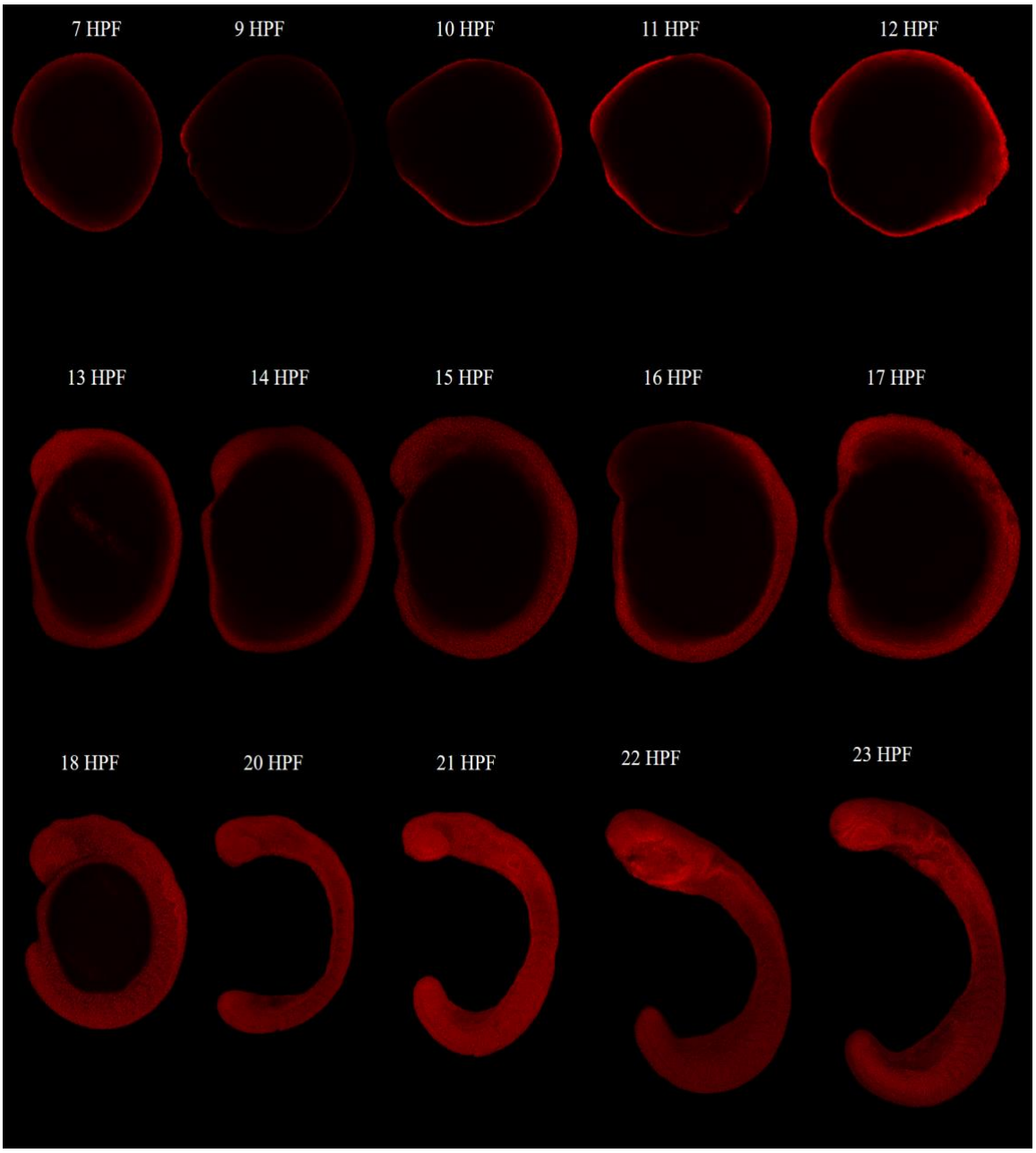
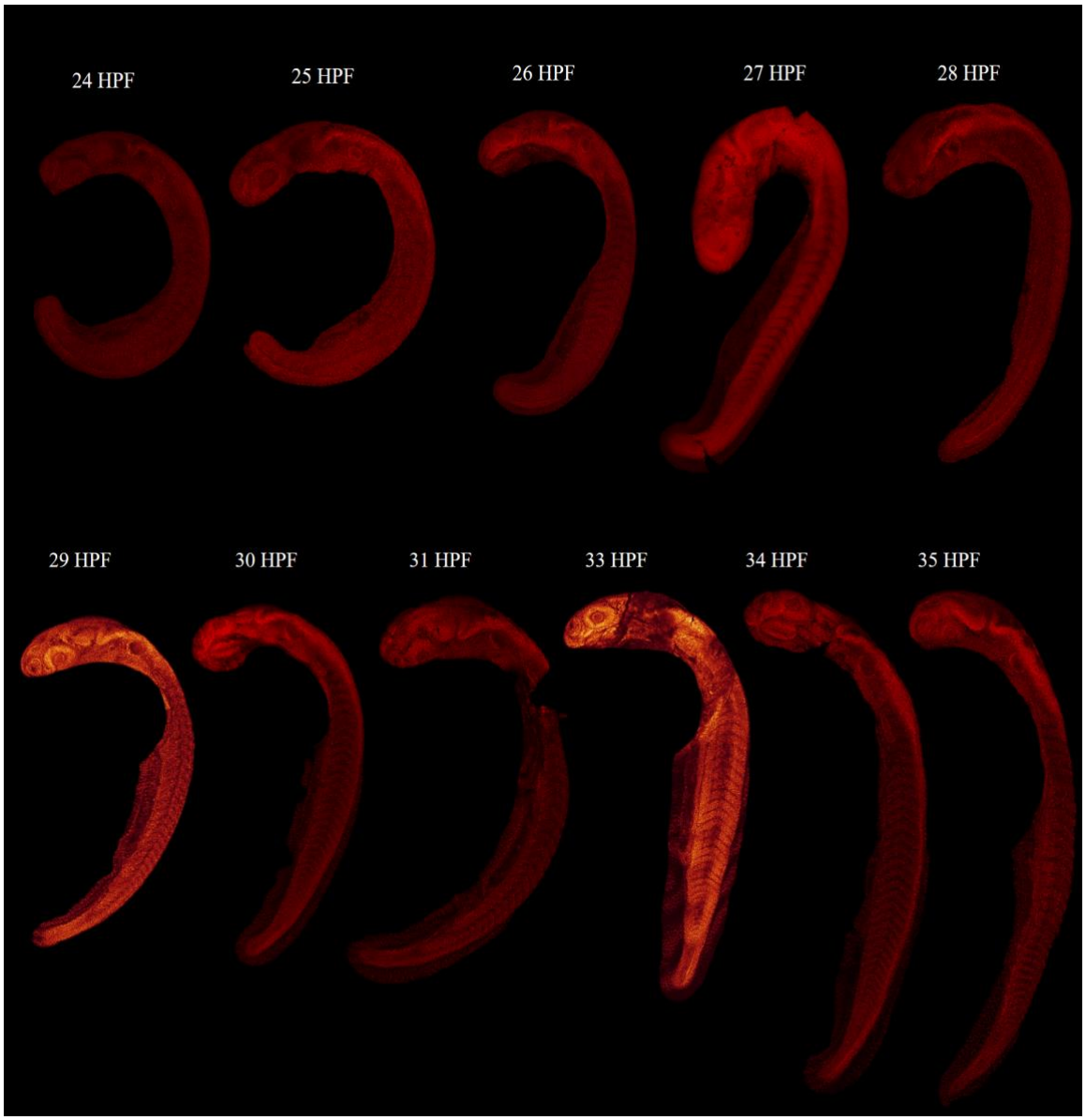
Appendix

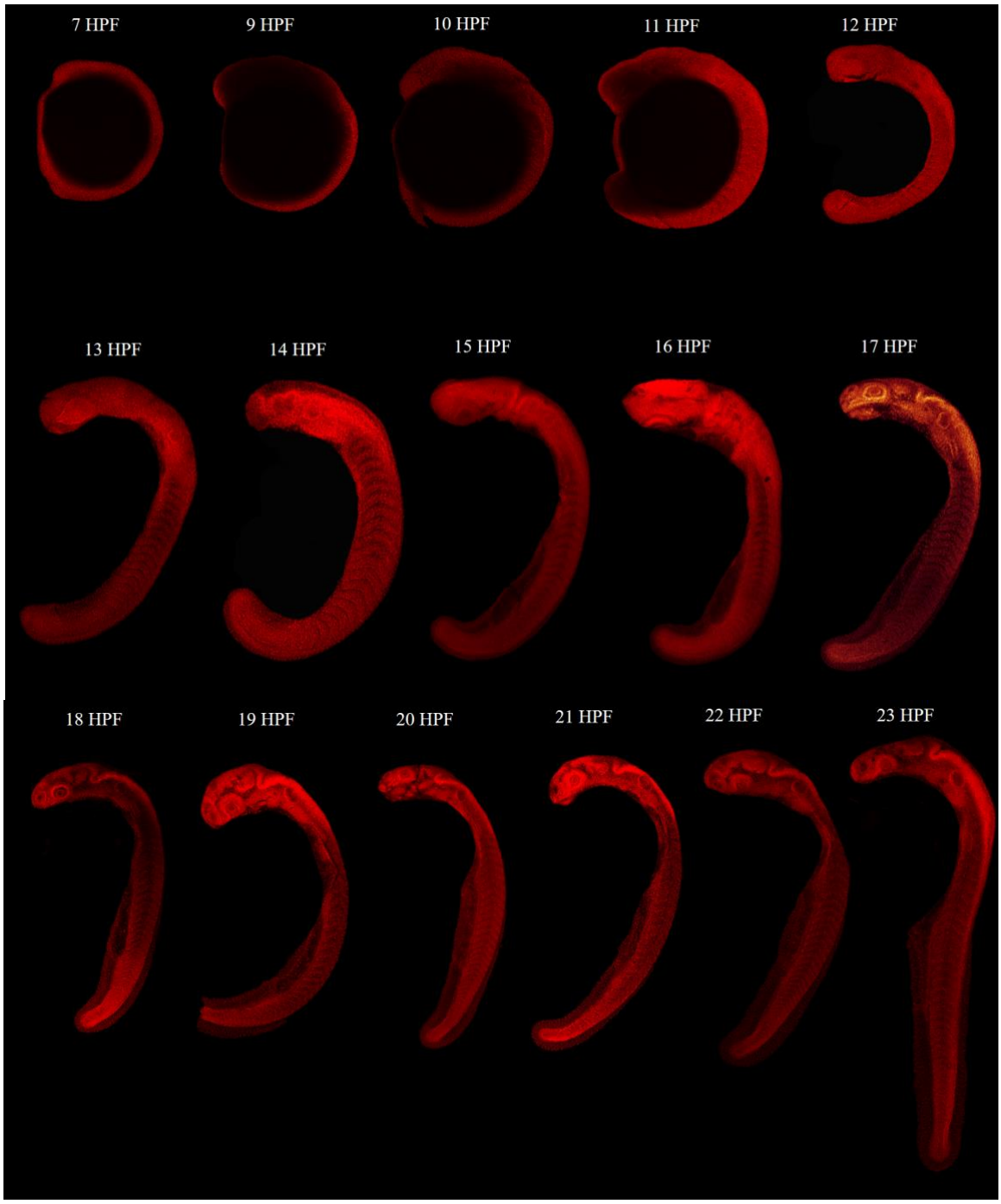
Appendix S1. Developmental stages of *Astyanax mexicanus* at the three temperature treatments.

A)




24 HPF
25 HPF
26 HPF
27 HPF
28 HPF
29 HPF
30 HPF
31 HPF
33 HPF
34 HPF
35 HPF

B)


7 HPF
9 HPF
10 HPF
11 HPF
12 HPF
13 HPF
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22 HPF
23 HPF

C)

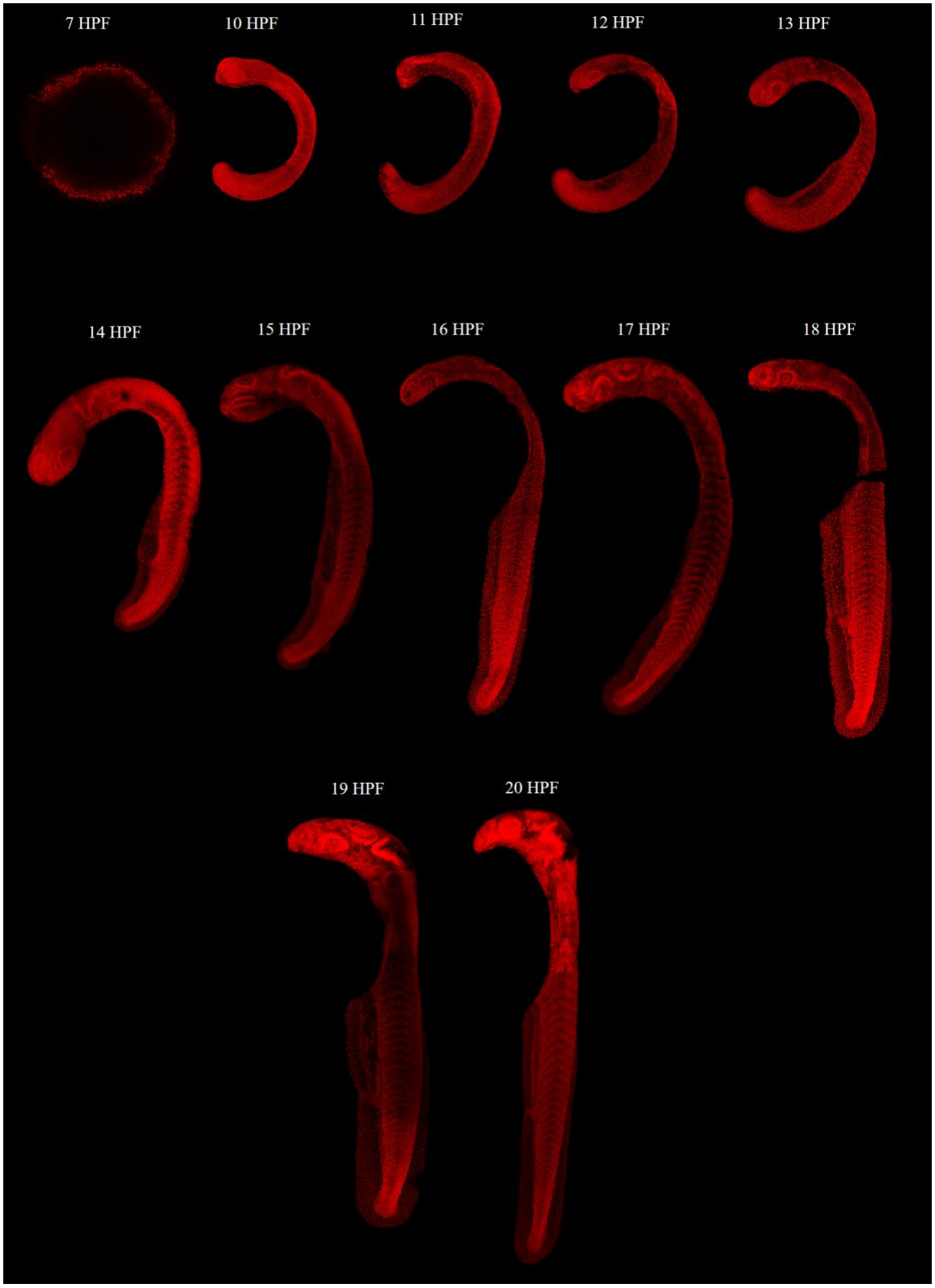


Figure S1: Developmental Rate of *Astyanax mexicanus* at 20ºC (A), 24ºC (B), and 28ºC (C). Figure shows differences of body growth rate, somite formation rate, and the reduction of unsegmented tail length among temperature treatments.

Appendix S2. The Impact of Temperature on Body Length and Unsegmented Tail Length When Accounting for Overall Developmental Rate by Somite Number

To examine whether temperature affects body length and unsegmented tail length once its general effect on total somite number is accounted for, I conducted two ANCOVAs with temperature as the experimental treatment, somite number as the covariate, the natural log of body length, and the natural log of the unsegmented tail length as the response variables. This allowed me to examine whether the body length and the unsegmented tail length differed between embryos of the same somite number at different temperatures. This analysis is complementary to the analysis presented in the results in which body length is used as the covariate to standardize by general developmental rate.

The full ANCOVA model for somite number was not significant for temperature and the interaction, but it was significant for body length (Table S1), so the temperature and the interaction of somite number and temperature were removed from the model by using a backwards stepwise regression. The impact of somite number on body length was significant (Table S2). As expected, as somite number increases, the body length elongates, but temperature has no effect on changing the body length when somite number was accounted for (Fig. S2 A)

Table S1: ANOVA table of the full ANCOVA model of body length. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (test statistic), P value (Probability value), SN (Somites Number), Temp (Temperature Treatments 20°C, 24°C, and 28°C), SN:Temp (The interaction between somite number and temperature), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
SN	1	0.55188	0.55188	487.7657	0.001
Temp	2	0.00026	0.00013	0.1164	0.8901
SN:Temp	2	0.00422	0.00211	1.8666	0.1578
Residuals	169	0.19121	0.00113		

Table S2: ANOVA table of the reduced ANCOVA model of body length. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (Test statistic), P value (Probability value), SN (Somite number), Temp (Temperature Treatments 20°C, 24°C, and 28°C), SN:Temp (The interaction between somite number and temperature), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
SN	1	0.55188	0.55188	487.86	0.001
Residuals	173	0.19570	0.00113		

The full ANCOVA model for UTL showed that somite number and temperature were significant, but the interaction between somite number and temperature was not significant (Table S3). A reduced model in which the interaction was removed showed that somite number and temperature remained significant (Table S4).

Unsegmented tail length was slightly larger in fish reared at 24°C than in fish reared at 20°C and 28°C, despite the fact that total somite number was the same between temperature treatments (Fig. S2 B)

Table S3: ANOVA table of the full ANCOVA model of unsegmented tail length. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (Test statistic), P value (Probability value), SN (Somites Number), Temp (Temperature Treatments 20°C, 24°C, and 28°C), SN:Temp (The interaction between somite number and temperature), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
SN	1	5.9272	5.9272	1016.6676	0.001
Temp	2	0.0449	0.0224	3.8502	.02311
SN:Temp	2	0.0137	0.0068	1.1715	0.31233
Residuals	174	1.0144	0.0058		

Table S4: ANOVA table of the reduced ANCOVA model of unsegmented tail length. *Df (Degrees of freedom), Sum Sq (The sum of squares), Mean sq (The mean squares), F value (Test statistic), P value (Probability value), SN (Somites Number), Temp (Temperature Treatments 20°C, 24°C, and 28°C), SN:Temp (The interaction between somite number and temperature), and Residuals.*

	Df	Sum Sq	Mean Sq	F value	P value
SN	1	5.9272	5.9272	1014.6900	0.001
Temp	2	0.0449	0.0224	3.8427	0.02326
Residuals	176	1.0281	0.0058		

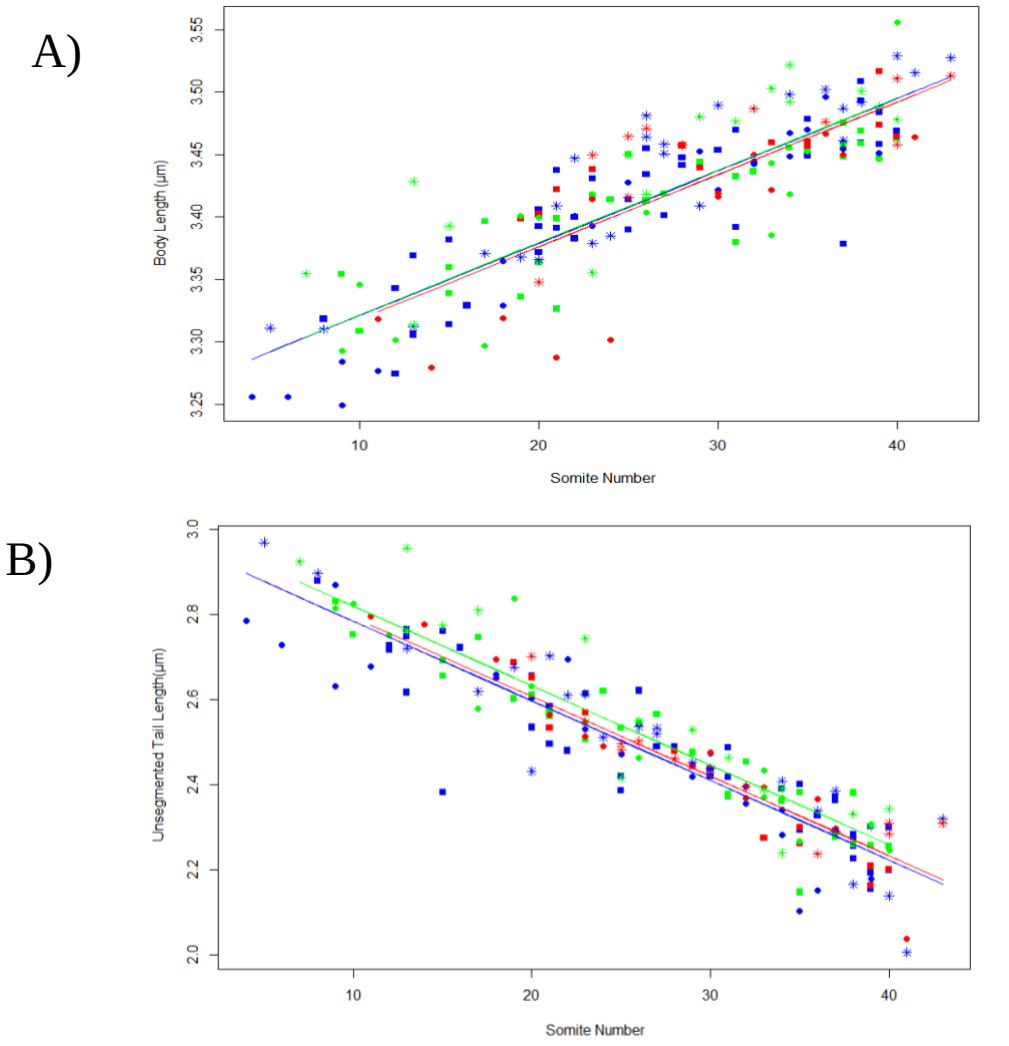
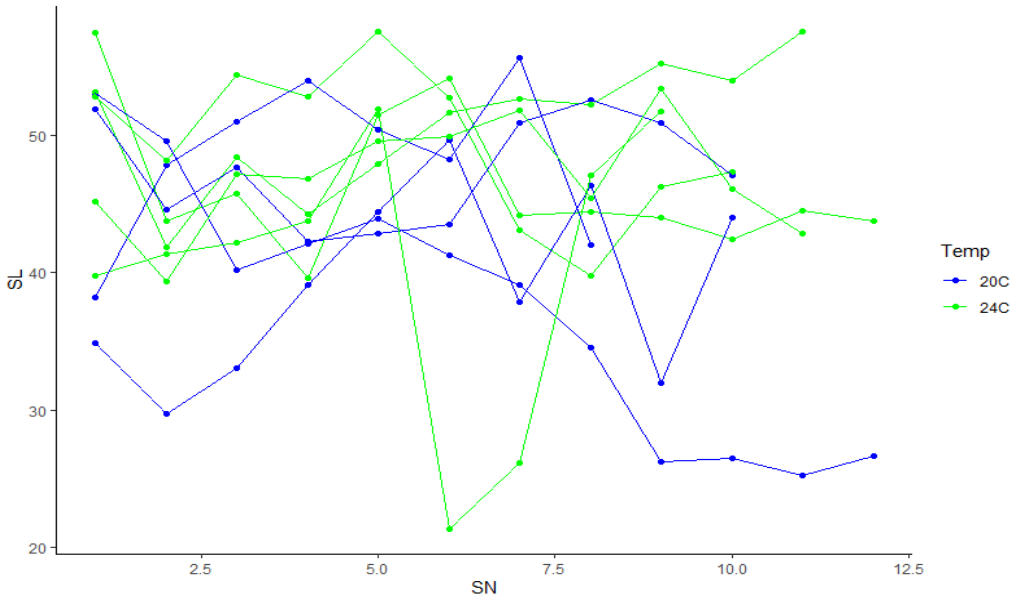


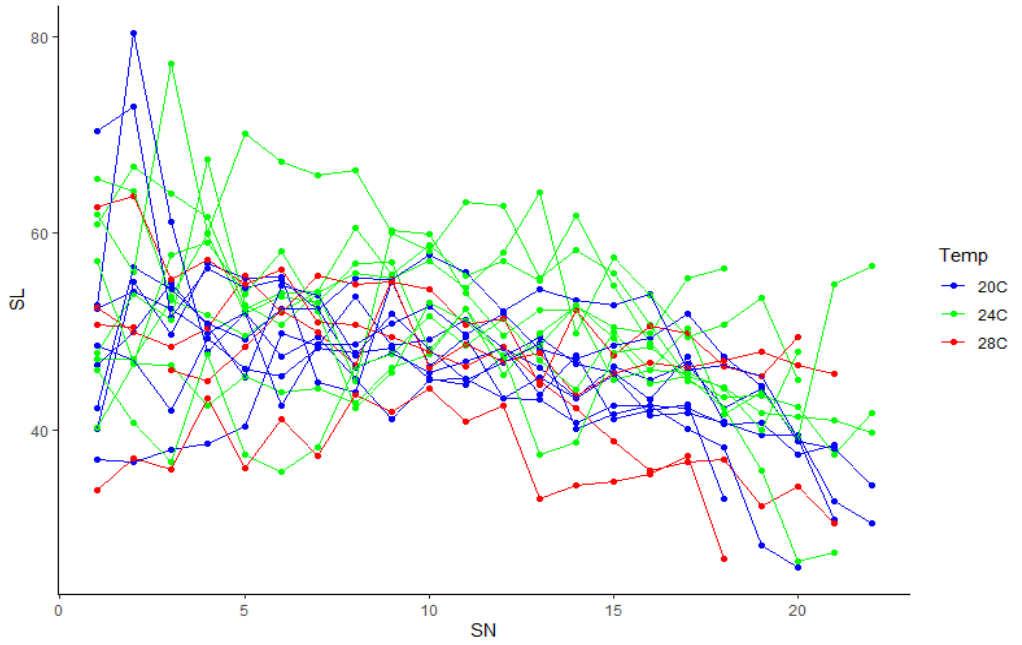
Figure S2: The effect of temperature on body length and unsegmented tail length when development rate is standardized using somite number as a covariate in embryos subjected to 20°C, 24°C, and 28°C temperature treatments. (A) Body length (in microns), (B) Unsegmented tail length (in microns) plotted as a function of somite number.

Appendix S3. Variation in Somite Length in Individuals of *Astyanax mexicanus* in the 10 (A), 20 (B), 30 (C), and 40 (D) somite stages.

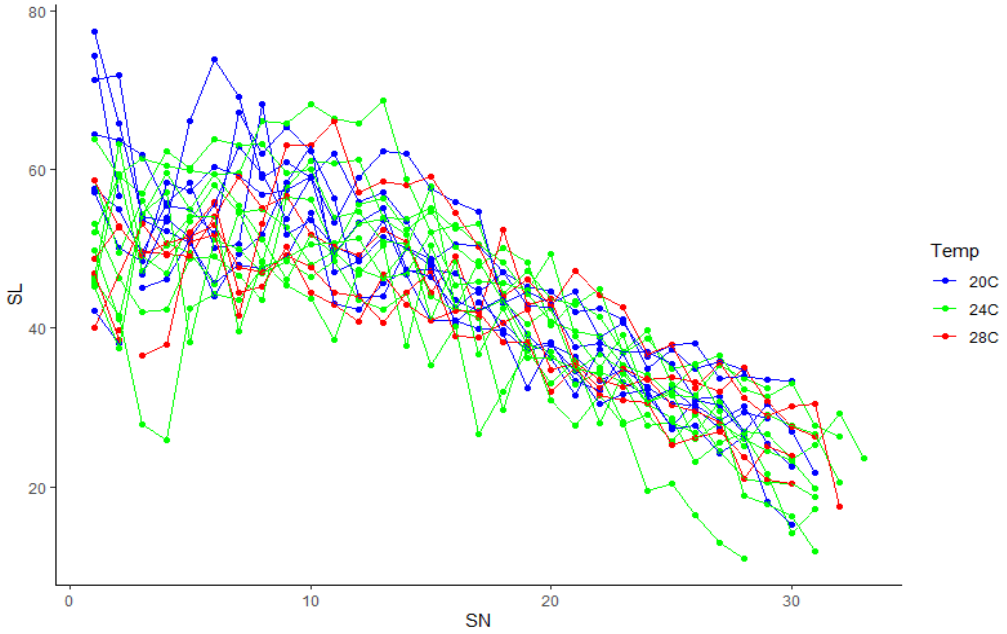
A)



B)



C)


80
60
40
20
SL
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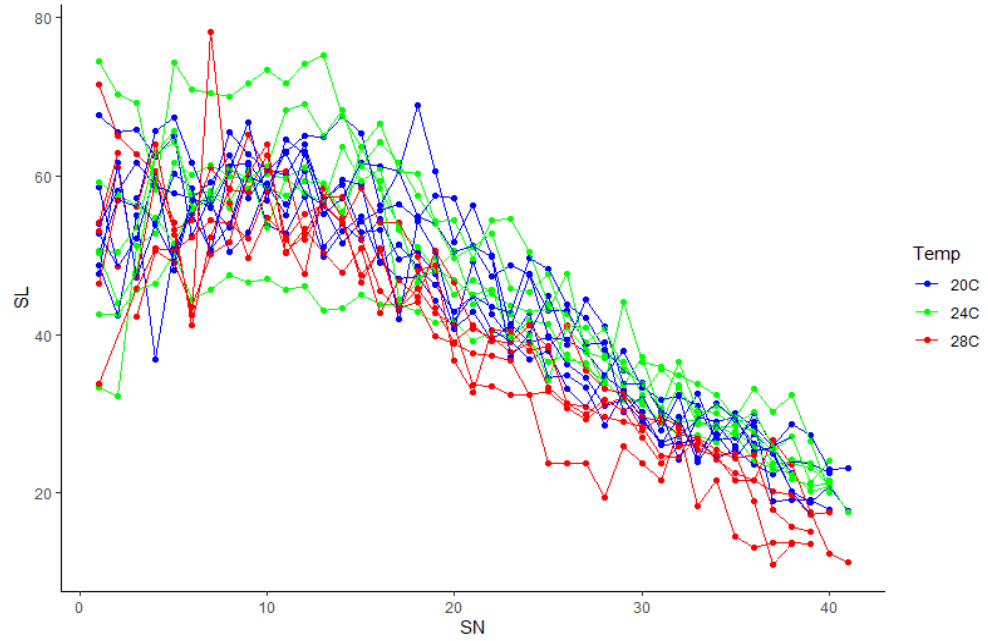
10
20
30
SN
Temp
20C
24C
28C

D)


80
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